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A STUDY OF THE REACTIONS OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES TO VARIOUS ALLERGENS

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THE purpose of this communication is to present the results of our attempts to evaluate a new diagnostic procedure described by Black[2] for the demonstration of sensitivity to allergens (antigens) of clinical significance in man. Since the simplicity and accuracy claimed for the test and its convenience so far as the patient is concerned are so great, it was felt that, if Black's work could be corroborated, conventional skin testing would soon become an obsolete procedure. It was believed, therefore, that this technique should be examined with extreme care.

The test involves the use of the patient's serum, living human polymorphonuclear leukocytes, and dried, powdered allergen. A preparation is made of these materials, as described under "Method," and the white cells are observed under the microscope. In a control preparation without allergen or in a preparation containing an allergen to which the patient is not sensitive, one should see polymorphonuclear leukocytes in their normal state, that is, with actively motile, ameboid movement, irregular outlines, and pseudopodia. If the preparation contained an allergen, this would be considered a negative test. According to Black, however, if the patient is sensitive to that allergen, the polymorphonuclear leukocytes will die; that is, they will become circular and will not exhibit

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TABLE I. DISTRIBUTION OF THE KNOWN ALLERGIC SUBJECTS ACCORDING TO THE IN VITRO RESULTS OF THEIR SERUM-POLYMORPHONUCLEAR LEUKOCYTE-ALLERGEN REACTIONS WHEN TESTED WITH THE SPECIFIC ALLERGEN TO WHICH THE SUBJECTS WERE CLINICALLY HYPERSENSITIVE

CLINICAL SENSITIVITIES	RAGWEED	COW'S MILK	HOUSE DUST	DOG	PEANUT	EGG	CAT	MIXED FEATHERS	WHEAT	TOTALS	CONTROLS
Total patient sensitivities	9	1	6	2	2	1	1	1	1	24	24
Total tests	29	2	8	4	3	1	1	2	1	51	30
Number of patients with clearly inactive results	0	0	0	1	0	0	0	0	0	1	10
Number of patients with clearly active results	3	1	0	0	0	0	0	0	0	4	8
Number of patients with clearly inactive tests and clearly inactive control	4	0	1	0	0	0	0	1	0	6	—
Number of patients not possible to interpret	2	0	5	1	2	1	1	0	1	13	15
Number of patients with both clearly active and inactive results	1	0	3	1	0	1	0	0	0	6	1
Number of patients with positive skin tests	8	0	2	1	1	1	1	1	1	16	—
Number of patients with negative skin tests	1	1	4	1	0	0	0	0	0	7	—

ameboid motion, motility of any kind, changing outlines, or pseudopodia. This is considered a positive test for the particular allergen concerned. Death is believed to result from the reaction of the reagin in the serum with its specific antigen (allergen). The rapidity with which death occurs is said to be directly proportional to the clinical sensitivity of the patient to the allergen.

For the sake of clarity, we have avoided the use of the terms "positive" and "negative" to describe the results of our tests. Instead, we have used the terms "active" and "inactive" corresponding to the motility of the leukocytes in the test preparations. It should be remembered, however, that leukocytes may become inactive for reasons other than the possible effects of the allergen. Errors in technique may alter results, and there may well be other factors of which we are as yet unaware.

METHOD

Test subjects who had clear-cut clinical sensitivities to foods, animal danders, or pollen and who had not received hyposensitization injections were chosen. The antigens used are listed in Tables I and II. The patients ranged in age from 1 to 11 years. Test materials used were Hollister-Stier powdered dried antigens. The pollens were ragweed and timothy and were whole granules rather than extracts.

Slides and coverslips were always new; they were prepared by washing with soap, brush, and tap water and were dried with Wipette tissues. Later, slides and coverslips were specially prepared, according to the method used in cleaning glassware for tissue culture experiments at the University of Rochester Medical School.

Except for minor variations, we adhered to the method described by Black. A ring of petrolatum the size of a coverslip was placed on a cleaned slide. In the center of the ring we placed a drop of triple-distilled water; later, special mineral-free water was used instead. A minute amount of powdered antigen was added to the water from the tip of a toothpick. This mixture was allowed to dry.

Serum-leukocyte mixtures were prepared with capillary tubes containing dried heparin. Approximately five such tubes were filled from a puncture site on the patient's finger and were spun in a microcentrifuge. Each tube was then cut with a small metal saw at a point just below the buffy coat so that the serum and buffy coat could be expelled into a common pool on a previously cleaned slide. From this pool of white cells and serum, a small drop was placed on each of the previously cleaned coverslips. The coverslip was then inverted, placed over the petrolatum ring on the slide, and gently sealed. The completed slides were placed in an incubator at 37° C. for one and one-half hours. Finally, they were transferred to a warming box and examined under the microscope with the high dry lens.

The slides were read as follows: Fifty polymorphonuclear leukocytes were counted on each test preparation and were recorded as active, sluggish, or inactive. Those cells which were clearly ameboid in form were classified as active.

TABLE II. DISTRIBUTION OF KNOWN ALLERGIC SUBJECTS ACCORDING TO THE IN VITRO RESULTS OF THEIR SERUM-POLYMORPHONUCLEAR LEUKOCYTE-ALLERGEN REACTIONS WHEN TESTED WITH ALLERGENS TO WHICH THE SUBJECTS WERE NOT CLINICALLY SENSITIVE

CLINICAL SENSITIVITIES	RAGWEED	COW'S MILK	HOUSE DUST	DOG	PEANUT	EGG	CAT	MIXED FEATHERS	WHEAT	TOTALS	CONTROLS
Total patient nonsensitivities	4	9	3	3	3	4	0	7	3	36	36
Total tests	8	13	5	5	3	7	0	10	3	54	40
Number of patients with clearly inactive results	0	0	0	0	0	0	0	0	0	0	12
Number of patients with clearly active results	2	4	0	1	1	3	0	1	1	13	21
Number of patients with clearly inactive tests and clearly inactive control	2	2	1	0	2	0	0	1	2	10	—
Number of patients not possible to interpret	0	3	2	2	0	1	0	5	0	13	13
Number of patients with both clearly active and inactive results	0	0	0	0	0	0	0	0	0	0	2
Number of patients with positive skin tests	2	0	1	1	0	1	0	4	2	11	—
Number of patients with negative skin tests	2	7	2	2	3	3	0	3	1	23	—

Inactive cells were so classified if they had a nearly circular shape and were without movement or cytoplasmic or particulate streaming. Cells which fell between these two groups were classified as sluggish.

All tests were read without the investigator knowing whether the test was for an antigen or a control test. The serum of each patient (except in three instances) was tested against three to eight different antigens, each of which was present two to four times among the test preparations. Included were two to four controls without added antigen. All slides were numbered rather than labeled and neither observer knew which test (including controls) was represented by any given number. Each observer read all the slides separately, without knowledge of the opinion of the other observer. Thus, there were two to four entirely separate preparations and four to eight separate readings for each antigen tested. After the slides were read and recorded by number, the key was consulted to evaluate the results obtained. No changes were allowed.

RESULTS

A total of 186 tests are considered in this report, besides an additional twenty-seven tests in clinically nonsensitive patients. There were thirteen patients with twenty-four known sensitivities. Ages ranged from 1½ years to 11 years. Of these, eleven were boys and two were girls.

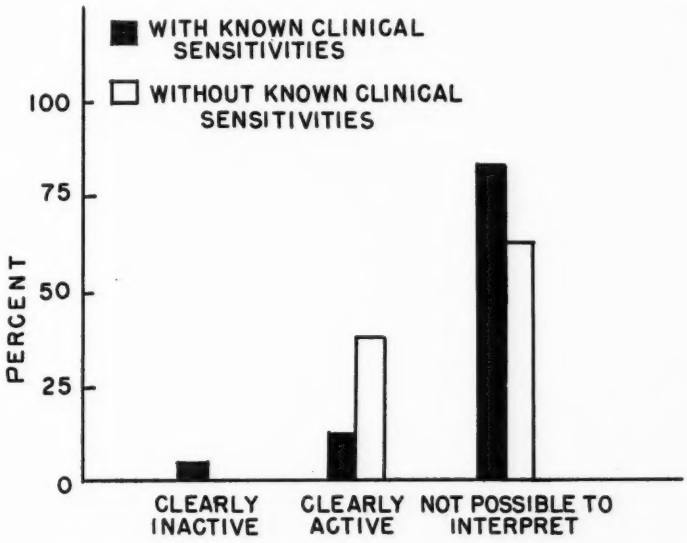

Fig. 1.—The percentage of total patients in the three categories of results (active, sluggish, inactive) for both sensitive and nonsensitive groups. It can be seen that the results are approximately equal for each classification.

The results are tabulated in Tables I and II, and the conclusions are summarized in Fig. 1. Fifty leukocytes were counted in each serum cell-antigen preparation and each was classified as active, sluggish, or inactive. In evaluating

our results, we discounted the sluggish cells, since the most accurate identification was of the clearly active and clearly inactive cells. According to Black,[3] 60 to 90 per cent of the leukocytes are normally active on the control preparations. Therefore, we interpreted a test as active if 60 per cent or more of the leukocytes were active. A test in which 60 per cent or more of the leukocytes were inactive was classified as inactive. No interpretation was attempted of results which did not fall into these two groups. Control readings were evaluated in exactly the same manner. Where more than one test was made with an antigen, the several values of both observers and of each preparation were averaged. Ten of the thirteen patients had two to four test preparations with each of the test antigens. Three patients were tested to each antigen only once. Thus, for most antigens tested, every preparation was read by both observers, giving four to eight separate readings.

Test slides were compared to their controls for final evaluation. Where the control was active and the test slide inactive, the test result was classed as clearly inactive. This would indicate clinical sensitivity, according to Black. If the test slide was active regardless of the state of the controls, it could be said to be uninfluenced by the test antigen, and was recorded as clearly active. This would indicate absence of clinical sensitivity, according to Black. Finally, if either the test slide or the control contained an approximately equal number of active and inactive cells, the result was not possible to interpret.

Fig. 1 lists the results of tests with a tigens to which the patients were clinically sensitive. It can be seen that of twenty-four patient sensitivities (some patients were clinically sensitive to more than one antigen), only one sensitivity (4 per cent) was detected by the test, four sensitivities (seventeen per cent) were missed by the test, and in nineteen sensitivities (eighty per cent) no interpretation could be made. In the group in which no interpretation could be made, one-third had inactive test results, but also had inactive controls, while two-thirds had approximately equal numbers of active and inactive results on either the test slide or the control, or both. In six instances (twenty-five per cent of the total patient sensitivities), there were some preparations which were active and some preparations which were inactive. These are listed separately.

When the same group of patients was tested with antigens to which there was no known clinical sensitivity, we obtained the results listed in Table II. There were no patients whose leukocytes were clearly inactive. Thirteen tests (36 per cent of the total) were clearly active, while twenty-three tests (64 per cent of the total) could not be interpreted.

The two tables do not represent controls for each other, but it appears that there is a random distribution of results among the various antigens and their controls. The results of these two tables are compared in the bar graph (Fig. 1).

Figs. 2 and 3 show an example of the leukocytes seen in 33 per cent of the patients clinically sensitive to ragweed pollen. Two leukocytes are noted by the black arrows, and are quite near a ragweed pollen grain (dotted arrow). It can

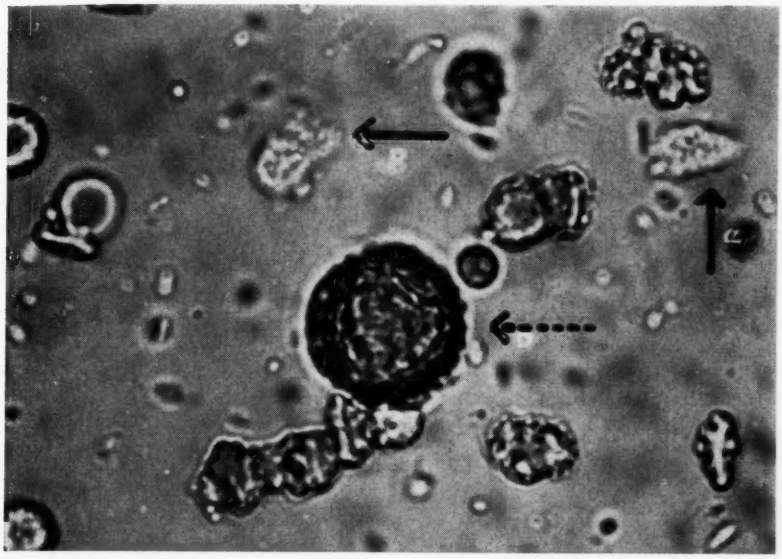

Fig. 2.—Photomicrograph of a test preparation from a ragweed-sensitive patient prepared as described in the text. A ragweed pollen grain (dotted arrow) can be seen in close proximity to two motile, live, active leukocytes (black arrows). The other objects are red cells in various stages of crenation, platelets, and bacteria. The liquid medium is the patient's serum. (Magnification, ×1,000.)

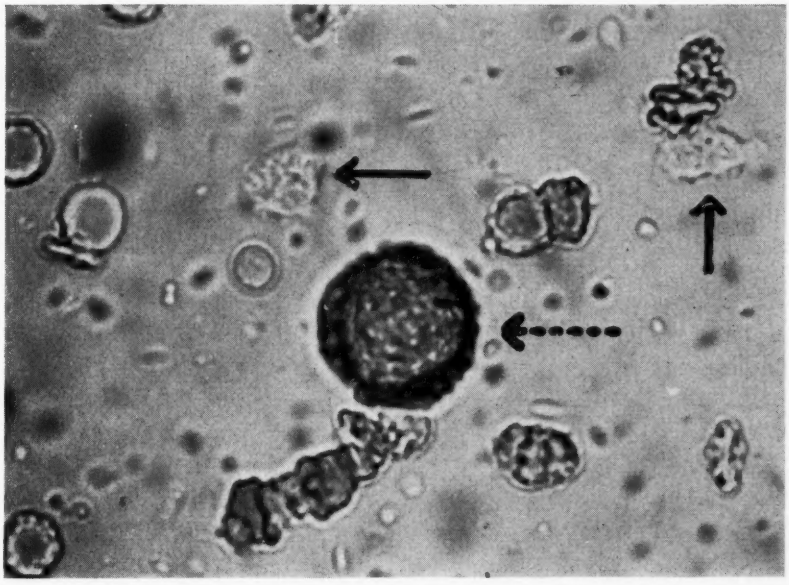

Fig. 3.—Photomicrograph taken ten seconds after the one in Fig. 2. shows motile changes in the two leukocytes, which have moved in space slightly and have characteristically changed in form as a result of their ameboid action. No dead white cells are seen in either photograph. The activity of the leukocytes in the presence of the pollen granule was much more striking in the original preparation than is demonstrated in the photograph. (Magnification, ×1,000.)

be seen that they are distinctly motile; that is, they are irregular, have pseudopods, and are motile as can be seen by comparing Fig. 2 with Fig. 3. These white cells are normal, motile, active leukocytes, and are what one would expect to see in a nonsensitive serum. Yet this patient is clinically sensitive to ragweed pollen, and the leukocytes are moving actively in close proximity to a ragweed pollen grain after one and one-half hours of incubation with it. This is a clearly active result, and its occurrence in three of nine sensitive patients is a major reason for concluding that the validity of the test is open to question.

Finally, it should be mentioned that preparation and examination of fifteen separate test preparations required about three hours for each observer.

DISCUSSION

A few comments on our method should be made. An incubation period of one and one-half hours was arbitrarily chosen. In Black's original report, the tests were often positive in fifteen minutes, usually within thirty minutes, and almost always within fifty minutes. It is possible that low reagin titers might have been present in some patients and that one and one-half hours was an inadequate incubation period. This appears unlikely in view of the clear-cut clinical sensitivities, as well as the presence in most cases of positive skin tests.

It is difficult to explain why some slides prepared from the serum and cells of any given patient should contain active cells, while others contained mostly inactive cells. Several factors which might have been responsible for this were studied. According to Sieracki,[7] leukocytes remain viable in vitro from eight hours to several weeks, under proper conditions. Therefore, it would appear that the nonsensitive cells and the control cells should have remained alive for the time interval studied. One nonallergic patient was studied on two occasions with fifty preparations, using ragweed pollen, beef antigen, and controls. This study gave similar inconstant results, as previously described for the other patients. A comparison was made between cells centrifuged at 10,000 r.p.m. for thirty to sixty seconds, and cells spun at 1,200 r.p.m. for fifteen minutes, as was originally suggested. No difference was noted. Furthermore, there were active and inactive cells spread throughout any given series of tests, indicating that spinning apparently had no lethal effect. Glassware washed with soap and tap water was compared with glassware washed according to methods used in tissue culture work. No difference was noted in either case. No change was noted when the dried heparin in the microhematocrit tubes was replaced with ACD solution. The use of fresh toothpicks for stirring cell suspensions gave the same results as the use of glass rods. Slight variations in temperature were unimportant.

Finally, interpretation of the slides was often difficult. Inactive leukocytes and mononuclear cells could not be distinguished with certainty. Others have noted a similar problem.[4, 6] In addition, lymphocytes are often motile and ameboid themselves.[1] Vital staining with neutral red was of no help to us, and this procedure may be cytotoxic.[5] The phase microscope was used briefly to

see if it would help us differentiate lymphocytes from dead polymorphonuclear leukocytes, but it was of no help in this particular problem. Identification of live, motile leukocytes was definite and presented no problem for the reasons discussed previously.

Each test preparation was read by two independent observers, neither of whom knew whether he was examining an actual test or a control; nor did he know the decision of the other observer as to the reading. This procedure allows one to measure agreement of interpretation. Of 213 tests, there was agreement in 64 per cent of the readings and failure to agree in 36 per cent of the readings. That two observers did not agree on one-third of the test slides must be attributed either to lack of special training or to inherent difficulty in classifying the leukocytes. Since six months' experience did not materially improve the index of agreement, we tend to favor the latter possibility.

Our results do not confirm the value of the leukocyte-serum-antigen test for allergy. While it is true that the test results are clouded by an unexplained inactivity in some of the control slides, one might expect to see a favorable trend in the results if the tests were valid. No such trend appears in the bar graph (Fig. 1) which summarizes our findings. It may be noted that, while most of the allergens used in the original study were foods, this report includes only five patient sensitivities to foods, of a total of twenty-four patient sensitivities. It is likely to be more difficult to be clinically certain of food sensitivities than of pollen or epidermoid sensitivities. However, of the five patients with food sensitivities, none showed clear inactivity of leukocytes, while one 1½-year-old boy who repeatedly developed bloody diarrhea on ingestion of cow's milk had a clearly active leukocyte test.

SUMMARY

1. Use of the serum-leukocyte-antigen test described by Black clearly revealed sensitivity in only one (4 per cent) of twenty-four patient sensitivities, while four patient sensitivities (17 per cent) were completely missed by the test.

2. In approximately 70 per cent of the test results, definite interpretation could not be made.

3. There was close agreement by independent observers on only two-thirds of the test preparations.

4. Some of the control slides showed an inactivity of leukocytes which was not understood.

5. The test is time consuming and not suitable for routine use by the practicing allergist. Its validity is seriously questioned.

REFERENCES

1. Bessis, M.: Cytology of the Blood and Blood-Forming Organs, New York, 1956, Grune & Stratton, p. 498.
2. Black, J. A.: A New Diagnostic Method in Allergic Disease, Pediatrics **17:** 716-724, 1956.
3. Black, J. A.: Personal Communication.

4. Millman, S., and Millman, M.: Preliminary Studies on an in Vitro Test for the Detection of Bacterial Allergies, The Letters of the International Correspondence Society of Allergists, Series XVIII: 69-76, 1955.
5. Richter, K. M.: Studies on Leukocytic Secretory Activity, Ann. New York Acad. Sc. **59**: 863-895, 1954-55.
6. Scherago, M., Partin, J., and Hall, H.: A Study of the Sensitivity of Human Leukocytes to Tuberculin and Other Bacterial Products by the Blatt, Nantz, and Rehm Technique, Ann. Allergy **15**: 1-12, 1957.
7. Sieracki, J. D.: The Neutrophylic Leukocyte, Ann. New York Acad. Sc. **59**: 690-705, 1954-55.

THE APPLICABILITY OF IN VITRO TESTS IN THE MEASUREMENT OF NEUTRALIZING ANTIBODY FOR RAGWEED POLLEN EXTRACT

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FIXATION of guinea pig complement (C′) by serum obtained from a few pollen-sensitive persons, both untreated and treated, was described over forty years ago.[1, 2] This phenomenon also appears to occur when the patient's unheated serum is used as the source of C′,[3-6] but here the specificity of fixation may be questioned, as satisfactory control of the anticomplementary properties of the antigen is not possible. Inhibition of complement fixation in a mixture of rabbit antiserum and pollen extract by human serum containing blocking antibody has also been described.[7]

The statement that "... for practical purposes and with the usual methods it can be said that precipitins are not found in atopic conditions"[8] is substantiated by the results obtained by most investigators during the past forty years or more with the usual interfacial and precipitin tests. However, special techniques, such as inhibition of precipitation[9] and the collodion particle technique,[10-12] have been used to detect antibody in sera of treated pollen-sensitive patients. In two studies[11, 12] in which the latter technique was employed it was found that sera from normal persons and from untreated ragweed-sensitive patients also agglutinated the sensitized collodion particles. Agglutination has been obtained, as well, with the tanned erythrocyte technique of Boyden and a "red cell linked antigen test."[13-15] Bands of precipitates were observed in a semisolid medium in tests with human serum and timothy pollen extract.[16]

In only a few studies[7, 9, 15] has an attempt been made to establish a relationship between the titer of antibody measured by such in vitro techniques and the pollen-neutralizing or skin-sensitizing capacity of the human sera. It is the purpose of this study to investigate this relationship in more detail.

MATERIALS AND METHODS

Pollen Extracts.—Ten grams of ether-washed ragweed or timothy pollen was extracted with 100 ml. of extracting fluid containing bicarbonate buffer[17] followed by filtration through a Mandler filter candle of regular porosity. Both the total nitrogen (TN) and the nitrogen precipitable by phosphotungstic acid (PTA-N)[18] were determined for most of the extracts. One unit of extract was

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defined as 0.01 mcg. of TN or 0.01 mcg. of PTA-N. A portion of the extract mixed with an equal volume of sterile glycerin was kept for use in the neutralization tests and in most of the fixation tests.

Crude pollen extract (both with and without glycerin), dialyzed crude extract (both with and without glycerin), and the dialyzed solution of the precipitate obtained from crude ragweed pollen extract at –10° C. with 20 per cent ethyl alcohol were used in the tests for inhibition of complement fixation.

Fractions I and II[19] were prepared from crude ragweed pollen extract with 50 and 100 per cent saturated ammonium sulfate according to the method of Newell.[20] The solution of the precipitate obtained after five successive precipitations at pH 4.0 with fully saturated ammonium sulfate was twice precipitated at 50 per cent saturated ammonium sulfate (Fraction I) and that remaining in solution was twice precipitated at 100 per cent saturated ammonium sulfate (Fraction II). After resolution and dialysis, the solution of Fraction I contained 0.186 mg. total nitrogen per milliliter and 0.102 mg. PTA-N per milliliter and the solution of Fraction II contained 0.406 mg. total nitrogen per milliliter and 0.276 PTA-N per milliliter. In agar diffusion tests with rabbit antiragweed serum, Fraction I formed five bands and Fraction II formed three bands. As judged by reactions of identity,[21] two antigens were common to both fractions.

After almost three years of storage at –18° C., Fractions I and II were still highly active by skin test. Upon heating to 70° C. for one hour, the solutions of both gave precipitate and the activity of both fractions in the skin of pollen-sensitive subjects was reduced, the reduction in activity being at least three to four times greater in the case of Fraction I. Since the allergenic activity of Fraction I, but not of Fraction II, is known to be destroyed at 70° C.,[22] it would appear that this preparation of Fraction I contained relatively little of Fraction II.

Subjects and Sera.—Blood was drawn aseptically, in the fasting state when possible, from pollen-sensitive patients and from normal subjects (Table I) and was allowed to clot in sterile centrifuge tubes. The sera were stored either

TABLE I. SUBJECTS AND SERA

	FIXATION TESTS			INHIBITION TESTS		
	NOT INJECTED	INJECTED		NOT INJECTED	INJECTED	
		Normal Subjects				
Number of subjects	9	7		4	7	
		Preinjection	Postinjection		Preinjection	Postinjection
Number of sera	9	6	19	4	5	10
		Pollen-Sensitive Patients				
	Group I	Group II				
Number of patients	6	19		0	10	
		Preinjection			Preinjection	Postinjection
Number of sera	4	0			8	12
		Postinjection				
Number of sera	6	19				

at 4° to 6° C. or at −18° C. without filtration or addition of preservative. Sera obtained from normal subjects who received no injections of pollen extract and the preinjection sera from injected normal subjects will hereafter be referred to as A sera$_N$ and the postinjection sera as P sera$_N$. Likewise, sera from patients will be designated as A sera$_P$ and P sera$_P$.

Rabbit Antisera.—Rabbits were given an initial subcutaneous injection of 1 ml. of an emulsion containing two parts of pollen extract 1:20, three parts of Falba, and six parts of mineral oil containing 20 mg. per milliliter of killed *Mycobacterium butyricum*. One to two months later the first of several series of three or four intravenous injections of 1:20 pollen extract was also given. Bleedings, obtained over a period of nine to twelve months, were made following each series of such injections.

Complement Fixation.—Pooled serum obtained from the clotted blood of four or more guinea pigs, to which 8.5 per cent sodium chloride was added to preserve the complement (C′), was stored at 4° to 6° C. Washed sheep cells, which had been stored in modified Alsever's solution,[23] were sensitized with two units of rabbit antisheep lysin. The diluent for all reagents was Veronal buffer.[24] Although two 100 per cent units of C′ (C′H$_{100}$)* were used in a few tests, in most tests three or four 50 per cent units (C′H$_{50}$) were used. In each case the amount of C′ was small, namely, 0.12 to 0.32 ml. of a 1:100 dilution of guinea pig serum. The C′H$_{50}$ unit was determined on the day the tests were set up. This was done essentially according to the technique described by Kent and associates,[25] either with the Klett-Summerson photoelectric colorimeter or with the Coleman junior spectrophotometer.

The highest concentration of pollen extract used was one-half that concentration which was anticomplementary. The greatest amount of serum which could be tested for fixing capacity was likewise limited by the anticomplement activity of the serum. An outline of a complement fixation test as done in this study is shown in Table II. A few sera were not heated before they were tested, but most of the tests were done with sera heated to 60° C., since in general, it was found that samples of sera heated to 60° C. were less anticomplementary than those heated to 56° C.

Inhibition of Complement Fixation.—This was done by a modification of the technique described by Rice.[26] The rabbit antisera, used in one-fourth the anticomplementary amount, fixed three to twelve C′H$_{50}$ units completely with four or more dilutions of ragweed pollen extract. The antitimothy serum fixed three C′H$_{50}$ units completely with three or more dilutions of timothy pollen extract, but with six C′H$_{50}$ units fixation was only 75 per cent. An outline of a representative test with crude glycerinated ragweed pollen extract is given in Table III. The human sera were diluted from 1:5 to 1:100, the dilutions usually employed being 1:10, 1:20, and 1:40. A reduction of 50 per cent or more in complete fixation was considered significant.

*One 100 per cent unit (C′H$_{100}$) is defined as that giving complete lysis of sensitized red cells.

TABLE II. REPRESENTATIVE COMPLEMENT FIXATION TEST WITH HUMAN SERUM

TUBE NO.	1	2	3	4	5	6	7	8	CONTROLS: SERUM (ML.)		ANTIGEN (ML.)			SYSTEM (ML.)			LYSIN (ML.)
Dilution of ragweed pollen extract	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280			1:2.5	1:5	1:10				
Volume (ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0	0.1	0.1	0.1	0	0	0	0
Three guinea pig $C'H_{50}$ units contained in 0.1 ml.																	
Volume (ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.066	0.033	0
P $serum_P$, Wa 1:5 volume (ml.)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0	0	0	0	0	0	0
Veronal buffer volume (ml.)	0	0	0	0	0	0	0	0	0.1	0	0.1	0.1	0.1	0.2	0.24	0.27	0.3
All tubes were held for 18 to 24 hours at 4° to 6° C.; 0.2 ml. of a 1 per cent suspension of sensitized sheep cells was added to each tube, following which the tubes were held at 37° C.; they were removed as soon as the serum and antigen control had cleared completely or after the elapse of 30 minutes																	
Per cent hemolysis	100	100	100	75	0	0	0	25	100	100	>90	100	100	100	75	50	0
Degree of fixation	0	0	0	1	4	4	4	3	0	0	Tr	0	0				

TABLE III. REPRESENTATIVE COMPLEMENT FIXATION INHIBITION TEST

TUBE NO.	1	2	3	4	5	6	7 SERUM (ML.)	8 SERUM (ML.)	CONTROLS * ANTIGEN 1:10	ANTIGEN 1:20	ANTIGEN 1:40	FIXATION 1:20	FIXATION 1:40	FIXATION 1:80	FIXATION 1:160	FIXATION 1:320	FIXATION 1:640	SERUM (ML.)	
Dilution of ragweed pollen extract	1:20	1:40	1:80	1:160	1:320	1:640													
			(Volume in ml.)																
PTA-N units/ 0.2 ml.	0.2	0.2	0.2	0.2	0.2	0.2	0	0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2		
	120	60	30	15	7.5	3.8													
P $serum_N$, Da_6 heated 30′, 60° C. Dil. 1:10, 1:20, 1:40†	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0	0	0	0	0	0	0	0	0	0
Veronal buffer	0	0	0	0	0	0	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0	0
2 to 4 Hours or 18 to 24 Hours at 4° to 6° C.																			
3 guinea pig $C'H_{50}$ units in 0.4 ml.	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Rabbit antiragweed serum diluted 1:160	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0	0	0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2
Veronal buffer	0	0	0	0	0	0	0	0	0.1	0.1	0.1	0	0	0	0	0	0	0.3	0.2
18 to 24 Hours at 4° to 6° C.																			
Sensitized cells, 2 per cent suspension	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
30 Minutes at 37° C.																			
Results of Test ‡																			
Da_6 1:10	2	1	4	4	Tr§	0	Tr	Tr	0	0	0	1	1	4	4	4	4	0	0
1:20	0	1	4	4	3	0	0	Tr											
1:40	0	1	2	4	4	1	Tr	Tr											

*System and lysin controls were included as in Table II.
†There was a series of Tubes 1 to 8 for each of the three dilutions of Da_6.
‡Results of the test are given in terms of degree of fixation.
§Trace of fixation.

Tests for Precipitins.—Approximately 0.05 ml. of serum in a small tube (3 mm. inside diameter) was overlaid with approximately 0.2 ml. of pollen extract in twofold serial dilutions in physiologic saline ranging from 1:10 or 1:20 to 1:320. If no precipitation at the interface was seen after two to four hours at room temperature, the tubes were stored for twenty-four or forty-eight hours at 4° to 6° C., and readings were again made. The contents of the tubes were then mixed, examined for precipitate and stored for six days in the refrigerator, whereupon final readings were made.

Precipitin tests were also done in a semisolid medium.[27] Tubes were prepared containing unglycerinated pollen extract, either undiluted or diluted 1:2 to 1:32 in the bicarbonate buffer solution. These dilutions were overlaid on a mixture of human serum diluted 1:2 with 0.6 per cent agar. Likewise, tubes were prepared containing the subject's serum diluted 1:4 with the buffer solution overlying mixtures of unglycerinated pollen extract, in final dilutions of 1:2 to 1:32 with 0.6 per cent agar. Readings were made during a period of fifteen days.

Skin-Sensitizing and Neutralizing Activity.—Tests for skin-sensitizing activity were done in the skin of normal human subjects by the usual method of passive transfer. To determine neutralizing activity, mixtures containing 0.1 ml. of serum, 0.1 ml. of pollen extract, suitably diluted, and 0.2 ml. of sterile physiologic saline were held at 4° to 6° C. for eighteen to twenty-four hours. They were then injected intracutaneously into sites prepared twenty-four to forty-eight hours before by the intracutaneous injection of serum showing skin-sensitizing activity for extracts of ragweed or timothy pollen.[28] The reactions were read after the elapse of ten to fifteen minutes. One unit of neutralizing activity was defined as that amount which would inhibit one unit of extract.

RESULTS

Tests With Serum From Normal Subjects

Neutralizing and Skin-Sensitizing Activity.—The period of injection, the number of injections and the amount of pollen extract injected for each of seven normal subjects and the neutralizing activity of eighteen P sera$_N$ obtained from them are shown in Table IV. In the determination of neutralizing activity, the mixtures of serum and pollen extract often produced some reaction in unsensitized as well an in sensitized sites, making it difficult to determine end points accurately. Thus, it was sometimes difficult to decide whether a given serum contained 1,000 or 1,500 PTA-N units per milliliter of neutralizing antibody, and in some cases 1,000 or 2,000 PTA-N units per milliliter. Although neutral points might have been determined more precisely if the skin-sensitizing serum had been included in the mixture of serum and pollen extract, the procedure used was chosen because it required much smaller amounts of sensitizing serum. The neutralizing activity of the P sera$_N$ was not clearly related to the total dose of extract administered.

One milliliter of four A $sera_N$ (La, Ma, Te, We) failed to neutralize 100 PTA-N units, the smallest number tested. Both the A serum and the P serum from subject La contained skin-sensitizing activity demonstrable in a dilution of 1:20. The A sera from the other three subjects were not tested for neutralizing and skin-sensitizing activity; the P sera from these subjects had no skin-sensitizing activity.

TABLE IV. AMOUNT OF NEUTRALIZING ANTIBODY IN POSTINJECTION SERA, NORMAL SUBJECTS

SUBJECT	SERIES	PERIOD OF INJECTION (WEEKS)	NO. OF INJECTIONS	TOTAL PTA-N UNITS INJECTED	SERUM NUMBER	PTA-N UNITS OF ANTIBODY/ML.
Da*	1	9	14	579,800	1	<200
Da*	2	5	11	154,600	2	1,000-1,500
Da*	3	2	8	158,300	3	500-1,000
–	–	–	–	–	4	200
Da*	4	3 to 4	13	731,100	5	3-5,000
Da*	–	–	–	–	6	3-5,000
Fo*	1	9	14	517,100	1	<200
Fo*	2	5	10	126,300	2	500
Fo*	3	6 to 7	15	348,100	3	500
–	–	–	–	–	4	100
Fo*	4	6	16	450,400	5	1,500-2,000
Fo*	–	–	–	–	6	1,500-2,000
Sw*	1	22	23	762,000	1	1-2,000
Sw*	2	5 to 6	10	264,800	3	1,000
La	1	8	19	334,600	1	1,000-1,500
Ma	1	9	17	352,000	1	2-500
Te	1	10	21	631,300	1	1,500-2,000
We	1	7	15	205,500	1	1,000-1,500

*Intervals between series: Da—11, 7 and 29 weeks; Fo—11, 7 and 20 weeks; Sw—9 weeks. In subsequent tables and in the text, a subscript to the subject's initials will refer to the serum number as indicated in the sixth column.

Complement Fixation Tests.—Two A $sera_N$ were so anticomplementary that no conclusions could be reached regarding their fixing capacity. The results with thirteen A $sera_N$ were negative.

The samples of P $sera_N$, Da_1, Fo_1, Da_2, Fo_2* were not heated before they were tested for fixing capacity because of the possibility that heat might so alter the antipollen antibody that it would fail to give fixation. The results with Da_1 and Fo_1, tested in a volume of 1 ml. and with a fixation period of four hours at 4° to 6° C., were negative. Since both of these sera contained less than 200 PTA-N units per milliliter of neutralizing antibody, no further tests were done with them. The fact that serum Da_1 was later found to contain precipitin for ragweed pollen extract suggests that fixation would have occurred if it had been heated and used in a test in which the fixing volume was either 0.3 or 0.5 ml. and the period of fixation eighteen to twenty four hours at 4° to 6° C., as was done later with other P $sera_N$. The results obtained with Da_2 and Fo_2 are shown in Table V. The difference in the amounts of C′ present in unheated Da_2 and Fo_2 might explain the positive result with Fo_2, which contained very little C′, and the negative results with Da_2, which had greater hemolytic activity.

Under various conditions of testing (Table V) seven out of seventeen P $sera_N$ gave complete fixation with at least one dilution of ragween pollen extract

*For explanation of subscripts, see footnote to Table IV.

and Da_2, Da_3, and Fo_2 gave two zones of fixation. Only three sera failed entirely to give any fixation; two of these (Da_4 and Fo_4) were obtained after the elapse of twenty-nine and twenty weeks, respectively, following completion of the third series of injections of extract.

TABLE V. FIXATION OF GUINEA PIG C′ BY SEVENTEEN P SERA$_N$

SERUM	ML.	VOLUME FIXING	VOLUME FINAL	RAGWEED POLLEN EXTRACT*						SERUM CONTROL (ML.)			ANTIGEN CONTROL*
				30	60	120	240	480	960	0.04	0.08	0.16	30
Sw_1	0.04	0.8	1.0	0	0	0	0	0	0	0	0	–	0
				12	24	48	96	192	384				12
Sw_1	0.04	0.5	1.0	0	3	4	4	2	0	0	0	–	0
Sw_2	0.033 and 0.02	0.5	1.0	0	0	0	0	0	–	0	0		0
Da_2	0.04	0.5	1.0	0	0	0	2	2	0	0	0	–	0
Da_2	0.05†	0.5	1.0	2	0	0	3	2	0				0
Da_2	0.02†	0.5	1.0	0	0	0	0	0	3	0	0	–	0
Da_2	0.02‡	0.5	1.0	0	0	0	0	0	0	0	0	–	0
Fo_2	0.02‡	0.5	1.0	3	1	4	1	0	0	0	0	–	0
Fo_2	0.04	0.5	1.0	0	0	2	2	0	0	0	0	–	0
Fo_2	0.05†	0.5	1.0	1	1	1	2	0	0	0	0	–	0
				5	10	20	40	80	160	320	640	1,280				2.5	5	10	20
Fo_3	0.04	0.5	1.0	–	1	2	1	Tr	Tr	Tr	Tr	–	0	0	–	–	Tr	0	–
Sw_3	0.04	0.5	1.0	–	3	2	1	1	1	Tr	Tr	–	0	0	–	–	Tr	0	–
Da_3§	0.033	0.5	1.0																
Da_4	0.04	0.5	1.0	–	–	–	0	0	0	0	0	0	0	0		–	–	0	0
Fo_4	0.04	0.5	1.0	–	0	0	0	0	0	0	0	0	0	0	–	–	–	0	0
Da_5	0.04	0.3	0.5	4	0	0	0	0	–	–	–	–	0	0	–	–	0	0	–
Da_6	0.04	0.3	0.5	4	2	1	0	–	–	–	–	–	0	0	–	2	0	0	–
Fo_5	0.04	0.3	0.5	3	2	1	0	–	–	–	–	–	0	0		–	0	0	–
Fo_6	0.04	0.3	0.5	–	3	2	0	0	0	0	–	–	0	0		–	–	0	–
La_1	0.02	0.5	1.0	–	–	–	–	0	0	2	1	0	0	Tr	–	–	0	0	–
La_1	0.02	0.3	0.5	0	0	0	0	0	3	4	4	1	0	0	0	–	0	0	–
We_1	0.02	0.3	0.5	–	–	–	0	0	0	1	2	0	0	0	1	–	0	0	–
We_1	0.02	0.5	1.0	–	0	0	0	0	0	0	Tr	0	0	1	1	–	1	0	–
Ma_1	0.02	0.3	0.5	–	0	0	0	0	0	0	0	0	0	0	3	–	2	0	–
Ma_1‖	0.04	0.3	0.5	–	4	2	2	3	2	0	0	0	0	0	2	–	–	0	0
Te_1	0.02	0.3	0.5	–	–	0	0	1	1	0	0	0	0	0	1	–	2	0	–
Te_1‖	0.04	0.3	0.5	–	2	4	3	3	2	–	–	–	0	2	2	–	–	0	0

Key to Symbols: 4 = Complete fixation; 3 = 75 per cent fixation; 2 = 50 per cent fixation; 1 = 25 per cent fixation; Tr = trace of fixation; 0 = no fixation.

*Expressed as reciprocals of the dilutions of extract. When fixation and final volume were 0.8 and 1.0 ml., the pollen extract was added in a volume of 0.5 ml.; with fixing and final volumes of 0.5 ml., and 1.0 ml., the extract was added in a volume of 0.2 ml., and with fixing and final volumes of 0.3 and 0.5 ml., the extract was added in a volume of 0.1 ml.

†Serum heated at 56° C. for thirty minutes.

‡Serum not heated.

§Da_3, 0.033 ml. gave 50 per cent fixation with extract diluted 1:10 and 1:400. There was no fixation with 0.025 and 0.02 ml.

‖Six $C'H_{50}$ units were used in these tests.

(Unless indicated otherwise, sera were heated for thirty minutes at 60° C. before they were tested.)

Fractions I and II were used in fixation tests with four P sera$_N$ that had been stored at –18° C. for one to two years (Table VI). Fixation by these sera, especially with samples Da_3, Fo_5, and We_1, was greater in degree and extent than that observed with crude pollen extract (Table V). Serum Fo_5 was more anticomplementary than the other sera but there is no doubt that the fixation shown in Table VI was specific. The degree of fixation with Da_3 and Fraction I was not

so great as that with the other three sera and, in contrast to the other sera, Da_3 fixed significantly only with the 1:100 dilution of Fraction II. As stated previously, it was found in agar diffusion tests with rabbit-antiragweed serum that solutions of Fraction I contained five antigens, two of which appeared to be identical with two of the three antigens present in the solution of Fraction II. It is impossible to decide whether the fixation given by these sera with both fractions was due to common antigens or to different antigens.

TABLE VI. FIXATION TESTS WITH FRACTIONS I AND II AND SERUM FROM NORMAL SUBJECTS INJECTED WITH CRUDE POLLEN EXTRACT

	ANTIGEN								SERUM CONTROLS			ANTIGEN CONTROLS		
									1:3		UNDIL.			
SERUM*	5	10	20	40	50	100	200	400†	0.1	0.2	0.1	2.5	5	10†
Fraction I														
Da_3	2	2	2	3	3	1	0	–	0	Tr	1	Tr	0	0
Fo_5	2	3	4	4	4	4	3	–	Tr	3	4			
La_1	–	–	4	4	4	4	4	2	Tr	1	3			
We_1	–	–	4	4	4	4	3	1	0	1	2			
Fraction II														
Da_3	Tr	0	Tr	Tr	Tr	3	1	0				0	0	0
Fo_5	2	2	2	3	4	4	1	–						
La_1	–	–	1	4	4	4	4	2						
We_1	–	–	3	4	4	4	4	1						

Key to Symbols: 4 = 100 per cent fixation; 3 = 75 per cent fixation; 2 = 50 per cent fixation; 1 = 25 per cent fixation; Tr = trace of fixation; 0 = no fixation.

*0.1 ml. serum diluted 1:3 used in each instance.

†Reciprocals of antigen dilutions. Volume of dilutions used was 0.1 ml.

Specificity of Fixation.—Only one (Fo_3) out of sixteen sera tested with timothy pollen extract showed any degree of fixation. Since the serum control did not clear completely in this test, it is questionable whether the weak fixation observed was specific.

Effect of the Amount of Pollen Extract Injected Upon the Results in Fixation Tests.—Fixing antibody was present in the sera of five of the seven subjects (La, Ma, Sw, Te, and We) after one series of injections. The number of injections given these subjects varied from fifteen to twenty-three, and the total number of PTA-N units injected varied from 205,500 to 762,000 (Table IV). Weak fixation was obtained with sera from subjects Da and Fo after two series of injections totaling 733,800 and 643,400 PTA-N units of extract, respectively (Tables IV and V). With sera obtained from Da, Fo, and Sw after further series of injections, fixation occurred with lower dilutions of extract, but there was no increase in extent and no consistent increase in degree of fixation. It is perhaps pertinent that serum from later bleedings of rabbits receiving several series of injections usually fixed to a greater degree and with a larger number of serial twofold dilutions of extract than serum from the earlier bleedings.

Effect of Technical Procedures on Results in Fixation Tests

Effect of Volume.—Fixation volumes of 0.3, 0.5, and 0.8 ml. and final volumes of 0.5 and 1.0 ml. were compared in tests with six sera. In five out of six such tests, the smaller volumes favored fixation (see Table V for results with

TABLE VII. FIXATION OF GUINEA PIG C′ BY NINETEEN P SERA[P]

PATIENT	TREATMENT (YEARS)	DILUTION OF SERUM	RAGWEED POLLEN EXTRACT									SERUM CONTROLS (ML. OF SERUM DILUTION)			ANTIGEN CONTROL		
			5	10	20	40	80	160	320	640	1,280	2,560*	0.1	0.2	0.3	2.5	5	10*
Ba	13†	1:5	-	4	0	4	2	0	0	0	4	0	0	Tr	-	Tr	0	0
Ca‡	15†	1:5	2	1	0	0	0	0	0	0	0	-	0	2	4			
Ir	30†	1:2.5	0	0	0	0	1	1	1	2	0	0	0	0	0			
Mc	1§	1:5	2	Tr	0	0	0	0	0	0	0	-	0	0	0			
Pe‖	1¶	1:2.5	3	0	0	0	0	Tr	1	0	2	-	0	Tr	0			
Wa	1†	1:2.5	0	0	0	4	4	4	4	4	2	-	0	Tr	0			
		1:5	0	0	0	0	1	4	4	4	3	-	0	0	3			
		1:7.5	-	-	2	0	0	1	4	4	4	3						

Key to Symbols: 4 = complete fixation; 3 = 75 per cent fixation; 2 = 50 per cent fixation; 1 = 25 per cent fixation; Tr = trace of fixation; 0 = no fixation.

In Patient Br serum was too anticomplementary for a satisfactory test.

No fixation was observed in twelve patients. One of these twelve patients (Do) had received injections of both pollen extracts; her serum gave partial fixation with a 1:640 dilution of timothy pollen extract.

*Reciprocals of the dilutions of extract.

†Perennial treatment.

‡Patient received injections of ragweed pollen extract only, but serum also fixed with a 1:5 dilution of timothy pollen extract.

§Preseasonal treatment June, 1952, to Aug. 19, 1952.

‖Serum from Patient Pe, who had received injections of both ragweed and timothy pollen extract, gave 50 per cent or more fixation with 1:20, 1:40, and 1:80 dilutions of timothy pollen extract.

¶Preseasonal treatment June 10, 1952, to Aug. 26, 1952.

some sera). Positive results were obtained most often when the fixation volume was 0.3 ml. and the final volume was 0.5 ml. A relationship between maximal fixation and volume has been reported for other antigen-antibody systems.[29]

Effect of Duration of Storage of the Extract.—Three out of five samples of serum tested with a freshly prepared extract and with an extract that had been stored for a number of months at 4° to 6° C. fixed only with the fresh extract. It has been reported that more intense precipitation was obtained with fresh than with stored pollen extract in tests with rabbit antisera.[30]

Effect of Method of Preparing Dilutions of Extract.—From the results of a few tests in which closely spaced dilutions of extract were used rather than the usual serial twofold dilutions, it appeared that the amount of antigen giving fixation with a constant amount of human serum was more critical than with rabbit antiragweed sera which fixed completely with at least four twofold serial dilutions. It is possible that with most of the P sera N, a greater degree of fixation and fixation with a greater number of dilutions of extract would have occurred if more closely spaced dilutions had been used in all tests.

Tests With Serum From Pollen-Sensitive Subjects

Tests for Skin-Sensitizing and Neutralizing Activity.—The ten sera from patients in Group I (Table I) transferred sensitivity to normal human skin in dilutions of 1:2 to 1:300 and neutralized from 10 to 1,000 TN units per milliliter of pollen extract. The twenty sera used in the inhibition tests (Table I) transferred sensitivity in dilutions of 1:2 to 1:1,500. Ten of these sera, tested unheated, neutralized from 4 to 1,000 TN units per milliliter of pollen extract and seven neutralized from 75 to 200 PTA-N units per milliliter. Three sera were not tested for neutralizing capacity. The nineteen sera in Group II (Table I) were not tested for skin-sensitizing and neutralizing activity.

Complement Fixation.—Preliminary tests for complement fixation with four P sera$_A$ and six P sera$_P$ (Group I, Table I) were negative. These tests were done before it had been found that best results were obtained with sera heated at 60° C. for thirty minutes and when fixation was carried out in a volume of 0.5 ml. rather than 1.0 ml. Further tests were done in this manner with nineteen P sera$_P$* (Group II, Table I) obtained in August and September, 1952. The sera were diluted 1:2.5 and 1:5 and used with 0.1 ml. of progressively doubled dilutions (1:5 to 1:2,560) of crude glycerinated ragweed and timothy pollen extracts. Because the bloods were not drawn in the fasting state and therefore might have been somewhat anticomplementary, 4 instead of 3 $C'H_{50}$ units were used. The results are shown in Table VII. Three sera (Ba, Pe, Wa) exhibited more than one zone of fixation, and one of them (Wa) had exceptional fixing capacity. Serum from patient Ca, treated only with ragweed pollen extract, gave 50 per cent fixation with the 1:5 dilution of both pollen extracts. With this single exception, fixation appeared to be specific; that is, fixation occurred only with the

*Through the courtesy of Dr. Walter S. Burrage, seventeen of these samples were obtained from patients who were being treated in the clinic at the Massachusetts General Hospital, Boston, Massachusetts.

particular pollen extract given by injection to the donor of the serum. The results also indicated that development of fixing antibody was not related to duration of treatment.

Tests With Serum From Normal Subjects and From Pollen-Sensitive Patients

Complement Fixation Inhibition.—The results of fixation inhibition tests with five A $sera_N$, eight P $sera_A$, and twelve P $sera_P$ indicated that the inhibitory activity of serum heated for thirty minutes at 60° C. was related neither to the neutralizing activity nor to the skin-sensitizing activity of unheated serum. In brief, the results leading to the conclusion were as follows: two P $sera_A$ from a patient sensitive only to timothy pollen inhibited fixation by both rabbit antiragweed and rabbit antitimothy sera; a serum which sensitized normal human skin in a dilution of 1:50 was inhibitory, but other sera sensitizing in dilutions of 1:50, 1:100, 1:150, 1:500, and 1:750 failed to inhibit fixation; among three P $sera_P$ obtained from a patient sensitive to and treated with timothy pollen extract, all exhibiting equal neutralizing activity after heating, one failed to inhibit fixation, one inhibited in a dilution of 1:10, and the third inhibited in a dilution of 1:40. This serum, when diluted 1:10, also inhibited fixation by rabbit antiragweed serum.

Seventeen of the twenty-five sera tested for inhibitory activity with rabbit antipollen sera were used also in inhibition tests with either rabbit antiovalbumin or rabbit antihemocyanin. With these seventeen sera there was inhibition in one instance only, indicating that the inhibition of fixation described above may have been specific.

Portnoy and Sherman[7] reported that P $sera_P$ and P $sera_N$ containing 500 or more protein nitrogen neutralizing units per millimeter would inhibit fixation by rabbit antiragweed serum. Since the neutralizing activity of all but three of the sera used in the inhibition tests described above was less than 500 TN units per milliliter, additional tests were done with nine P $sera_N$, eight of which had neutralizing activity of 500 or more PTA-N units per milliliter and four A $sera_N$. Three of four A $sera_N$ were not inhibitory, but one (La_O, Table VIII) was inhibitory. The results with nine P $sera_N$ are shown in Table VIII. Four sera containing 500 or more PTA-N units per milliliter were not inhibitory, but one serum containing 200 units and one A $serum_N$ containing less than 100 units were inhibitory. When inhibition occurred it was not correlated with the number of neutralizing units of the serum. For exemple, 15 units of one serum (Da_6) inhibited fixation by rabbit antiragweed serum with 4 units of extract only, but 15 units or another serum, Fo_6, inhibited fixation with 4, 9 and 18 units of extract, and 15 units of a third serum, Te, did not inhibit with any number of units tested. Thus, no clear relationship between neutralizing and inhibitory activities was evident. The results, therefore, failed to confirm the findings of Portnoy and Sherman.[7] None of the ten sera inhibited fixation by rabbit antitimothy serum.

Precipitin Test.—The five A sera$_N$ (Da, La, Ma, Te, and We) that were tested gave negative results in the interfacial tests. Eight of twelve A sera$_N$, which included La, Ma, Te, and We, produced a band with one or more dilutions of ragweed pollen extract in agar-diffusion tests. Usually reactions occurred only with undiluted extract and extract diluted 1:2. The bands formed were very diffuse in character, and with four sera they were dense. Four of the eight A sera$_N$ that formed a band with ragweed pollen extract were tested with timothy pollen extract also. Three of the four (Ma, Te, and We) formed faint bands with one or more dilutions of this extract.

TABLE VIII. INHIBITION OF FIXATION BY RABBIT ANTIRAGWEED SERUM WITH HEATED P SERA$_N$

SUBJECT AND SERUM	NEUTRALIZING ACTIVITY OF SERUM (PTA-N UNITS/ML.)	UNITS OF NEUTRALIZING ACTIVITY ADDED	RAGWEED EXTRACT (PTA-N UNITS ADDED)			
			28-35	14-18	7-9	4
Da_3	500	5	0	0	+	-
Da_4	200	4	0	0	+	-
Da_6	3,000	30	0	0	+	+
		15	0	0	0	+
		7.5	+	0	0	+
Fo_3	500	5	0	0	0	-
Fo_6	1,500	15	0	+	+	-
		7.5	0	0	0	-
		3.8	+	0	0	+
Sw_3	1,000	20	0	+	0	-
La_0*	100	1	+	+	+	-
		0.5	+	0	0	0
		0.25	+	0	0	0
La_1	1,000	10, 5, 2.5	0	0	0	0
We_1	1,000	10, 5, 2.5	0	0	0	0
Te_1	1,500	15, 7.5, 3.8	0	0	0	0

Sera heated for thirty minutes at 60° C.

There was complete fixation by a combination of each of the concentrations of ragweed extract shown above and a constant amount of rabbit antiragweed serum in a volume of 1 ml.

+ = Fixation inhibited.

0 = Fixation not inhibited.

- = Not tested.

*Preinjection serum from La.

The results of interfacial tests with sixteen P sera$_N$ and sixteen P sera$_P$ and of agar-diffusion tests with twelve P sera$_N$ and fourteen P sera$_P$ are shown in Table IX. The amount of precipitate formed in the interfacial tests with six sera was small, but it was definite and control studies done with serum and diluent were negative.

It is interesting that two P sera$_N$ (Da_4, and Fo_4) and seven P sera$_P$ (Cr, Do, Ho, Hu, Le, Lv, and Ru, Table IX) gave negative results in the fixation tests but formed bands with at least two dilutions of extract in the agar-diffusion tests. With the exception of two sera (Cr and Ho), the bands were formed with stronger dilutions of extract than those used in the fixation tests. It is possible, therefore, that amounts of antigen and antibody, or their relative concentrations, were not in the optimal range in the fixation tests described earlier.

TABLE IX. SUMMARY OF IN VITRO TESTS

SERUM	NEUTRALIZING ACTIVITY (PTA-N UNITS/ML. x1,000)	C′-FIXATION TEST	INHIBITION C′-FIXATION TEST	INTERFACE TEST	AGAR-DIFFUSION TEST	
					RAGWEED EXTRACT	TIMOTHY EXTRACT
		Sera From Normal Subjects				
Da_1	<0.2	–	–	+	+ (3)	–
Da_2	1-1.5	+	–	0	+ (1)	0
Da_3	0.5-1	+	+	0	+ (2)	0
Da_4	0.2	0	+	0	+ (2)	0
Da_5	3-5	+	–	0	+ (3)	+ (1)
Da_6	3-5	+	+	0	+ (3)	+ (1)
Fo_1	<0.2	–	–	0	–	–
Fo_2	0.5	+	–	0	+ (2)	0
Fo_3	0.5	+	0	0	–	–
Fo_4	0.1	0	–	0	+ (2)	0
Fo_5	1.5-2	+	–	0	–	–
Fo_6	1.5-2	+	+	0	–	–
Sw_1	1-2	+	–	–	–	–
Sw_3	1	+	+	–	–	–
La_4	1.0-1.5	+	0	+	+ (2)	0
Ma_1	0.2-0.5	+	–	0	+ (3)	0
Te_1	1.5-2.0	+	0	+	+ (2)	0
We_1	1.0-1.5	+	0	0	+ (2)	0
		Sera From Pollen-Sensitive Patients				
Ba		+		0	+ (4)	0
Br		–		+	+ (2)	0
Ca		+ (R,T)		0	–	–
Cm		0		0	? (3)	0
Cr		0		0	+ (5)	+ (1)
Dv		0		–	–	–
Do		0 (R)		+ (R)	+ (1_3)	
		+ (T)		+ (T)		0
Fu		0		0	0	0
Ho		0		0	+ (4)	+ (3)
Hu		0		0	+ (2)	0
Ir		+		0	+ (4)	+ (5)
Ke		0		0	–	–
Kl		0		–	–	–
Le		0		0	+ (1)	–
Lv		0		0	+ (3)	+ (1)
Mc		+		–	+ (3_3)	+ (1)
Pe		+ (R,T)		0	+ (1_3)	0
Ru		0		0	+ (2_3)	–
Wa		+		+	+ (5)	0

P seraN: Da_1 to We_1, inclusive.
P seraP: Ba to Wa, inclusive.
0 = Absence of fixation, inhibition, or precipitation.
+ = Significant fixation, inhibition, or precipitation.
– = Not done.
Agar-diffusion: Numbers in parentheses indicate the number of dilutions with which a band was formed, and the subscripts indicate the maximal number of bands observed with any dilution of extract.

DISCUSSION

Since positive results in fixation tests were obtained more often when the volume in the tests was small, not only the amounts of antigen and antibody but also the concentrations of these reagents were important factors in fixation of guinea-pig C′ by human serum and ragweed pollen extract. The amount of extract required for fixation with a given amount of serum was critical, and if this amount was doubled or halved fixation often did not occur. As stated

before, perhaps fixation would have occurred more frequently and with more dilutions of extract if dilutions in all tests had been more closely spaced. The degree and extent of fixation occurring in tests with Fractions I and II (Table VI) suggest either that the crude extract contained an inhibitory component or that the amounts of antigen in dilutions prepared from these fractions were closer to those optimal for fixation. It is possible, therefore, that some failures to obtain positive results in vitro tests with crude pollen extract, in our studies and others, may not have been due entirely to the amount and character of the antibody in sera of pollen-sensitive subjects.

Comparison of the fixing and neutralizing activities was made by expressing the activity of both serum and pollen extract in PTA-N units. If identical antigen-antibody systems were concerned in fixation and neutralization of pollen extract, a reasonably constant ratio of PTA-N neutralizing units of serum to PTA-N units of pollen extract should be found in those tubes in which complete fixation occurred. The results with five sera (Table X) show the wide variation

TABLE X. RATIO* OF C′-FIXING TO NEUTRALIZING ACTIVITY OF P $SERA_N$† FOR RAGWEED POLLEN EXTRACT

SERUM	NEUTRALIZING ACTIVITY PTA-N UNITS/TUBE	PTA-N UNITS/TUBE	200	100	50	25	12.5	6.3		
Sw_1	40	Ratio		0.2	0.4	*0.8*	*1.6*	3.2	6.4	
		Degree of fixation		0	3	4	4	2	0	
Sw_3	40	Ratio		*0.2*	0.4	0.8	1.6	3.2	6.4	
		Degree of fixation		4	2	2	2	0	0	
Da_5	120	PTA-N units/tube	240	120	60	30	15	7.5	3.8	1.9
		Ratio	*0.5*	1	2	4	8	16	–	–
		Degree of fixation	4	0	0	0	0	0	–	–
La_1	20	Ratio	–	0.17	0.33	0.66	1.3	*2.7*	*5.3*	10.5
		Degree of fixation	–	0	0	0	3	4	4	1
Ma_1	8	Ratio	0.03	0.07	0.13	0.27	0.53	1.1	2.1	4.2
		Degree of fixation	4	3	2	2	3	0	0	0

*Ratio: Neutralizing activity of serum in PTA-N units divided by amount of extract in PTA-N units.

†Results with five sera showing complete fixation in one or more tubes.

in this ratio. Even though the determination of neutralizing activity was not precise, the observed variation from 0.03 to 5.3 in this ratio indicated a complete lack of correlation between the neutralizing and fixing capacities of the sera. For example, amounts of serum representing 8, 40, and 120 units of neutralizing activity fixed completely with 240, 200, and 240 units, respectively, of pollen extract, and in another test an amount of serum representing 40 neutralizing units failed to fix with 200 units of extract but did fix with 50 and 25 units. There was also variation in this ratio in mixtures in which lesser degrees of fixation occurred.

The multiple zones observed in fixation tests, both with some P $sera_N$ and with some P $sera_P$, and the formation of more than one band in agar-diffusion

tests with some of the latter sera are evidence that man, like the rabbit, can produce antibody to more than one of the five or eight antigens[27, 31] present in ragweed pollen. If different antigens and antibodies were reacting in the fixation and neutralization tests, no correlation between the results of the two tests would be anticipated. Moreover, several variables may be involved in the determination of the neutralizing activity of a given serum. Crude extract contains at least two allergens,[22] and both skin-sensitizing antibody and neutralizing antibody to more than one antigen may be present.[32, 33] It has been reported that fixation tests with rabbit antisera for a crude antigen containing several antigenic components did not yield a true estimate of the fixing capacity of such sera unless relative amounts of the antigens and their respective antibodies were present in suitable proportions.[34] The variables involved in the determination of both neutralizing activity and fixing capacity of sera are probably such as to preclude recognition of a constant relationship between these two activities, even if such a relationship existed. Similar considerations apply to the complement fixation inhibition test and to tests based on agglutination of red cells, bacteria, and collodion particles coated with antigen contained in complex mixtures. It is probable, therefore, as pointed out by others,[9] that any in vitro test with crude pollen extract is inapplicable to the detection and measurement of neutralizing activity.

Since positive results have been reported in tests with sera obtained from "untreated" normal subjects and collodion particles coated with crude ragweed pollen extract,[11, 12] it is interesting that eight of twelve such sera examined in this study produced a band with one or more dilutions of ragweed pollen extract, and three out of four of these sera also produced faint bands with one or more dilutions of timothy pollen extract in agar-diffusion tests. Similarly Frandsen and Samøe-Jensen,[35] using an agar-diffusion technique, also observed precipitates with extracts of one or more of eight different allergens and nine out of ten sera from normal subjects. It does not seem improbable that normal subjects, exposed as they are each year to airborne pollens, could produce antibody to one or more of the antigens in pollen. No relation was seen between antibody detected in the interfacial and agar-diffusion tests and clinical sensitivity to pollen or to the neutralizing activity of P $sera_N$. Indeed, such a relation to the latter was clearly lacking, since P $serum_N$ (Da_1) having less than 200 PTA-N units per milliliter of neutralizing activity gave more precipitate in the interfacial test than two P $sera_N$ having neutralizing activity of 1,000 and 1,500 PTA-N units per milliliter, respectively. Futhermore, nine other P $sera_N$ having 500 to 3,000 PTA-N units per milliliter gave no precipitate.

The results obtained in the complement-fixation inhibition tests in this study are not in agreement with those obtained in a similar and more extensive study by Portnoy and Sherman.[7] The inhibitory effect observed by Portnoy and Sherman was considered by them to be specific, for although heated normal sera and heated sera from patients treated with grass and tree pollen extracts failed to inhibit fixation by rabbit antiragweed serum, twelve heated sera having 500 or

more PTA-N units per milliliter of neutralizing activity for ragweed pollen extract did inhibit fixation. In terms of neutralizing activity of these twelve sera, 6.3 units in seven instances, 12.6 units in four instances, and 25 units in one instance were required to inhibit fixation by single optimal amounts of rabbit antiserum and ragweed pollen extract.[7] Since quantitative measurement of neutralizing activity is far from precise, a twofold difference in the amount of neutralizing activity required for inhibition would not be a serious discrepancy. A fourfold difference in amount of neutralizing activity required for inhibition, however, raises serious doubt that neutralizing antibody alone is actually measured by the inhibition technique.

The inhibition fixation test is based on the premise that the serum being tested does not fix complement. In the present study, eight of the nine P $sera_N$ tested for inhibition of complement fixation (Table VIII) fixed guinea pig C′ and it is possible that some of the sera tested by Portnoy and Sherman would also have fixed C′. It would be difficult to predict what effect the capacity to fix C′ would have upon the results of the inhibition test.

Certain details in procedure in the present study differed from those followed by Portnoy and Sherman in that the period of incubation and the incubationary temperature were different, fresh rather than stored sera were used, and the total volume in the test was larger. Portnoy and Sherman[7] showed in their tests that the inhibition which they observed was not due to increased hemolysis resulting from thermostable components of human serum. It is likewise improbable that these components were responsible for the inhibitory activity given by sera in the present study, for it was observed that hemolysis in many of the serum controls containing both heated human serum and rabbit serum was only about 90 per cent complete, whereas in serum controls containing rabbit serum alone there was 100 per cent hemolysis. Since normal and pollen-sensitive subjects can produce complement-fixing antibody when injected with ragweed pollen extract and since a nonspecific inhibitory factor may be present in some fresh human sera, titers obtained with the complement fixation inhibition test may not be reliable measures of antibody.

SUMMARY AND CONCLUSIONS

Sera obtained from normal subjects and from pollen-sensitive patients before and after a series of injections of ragweed pollen extract were studied for their capacity (1) to neutralize the skin-reactive components in pollen extract, (2) to fix complement with crude and fractionated pollen extract, (3) to inhibit fixation of complement by rabbit antisera for pollen extract, and (4) to produce bands of precipitate in semisolid media.

Satisfactory correlation could not be obtained between the tests for neutralizing capacity and any of the above in vitro tests for antibody. Possible reasons for failure to obtain a correlation are discussed. Major difficulties are that any antibody detected may not have neutralizing activity or that antibody for two or more antigens may be present. This latter possibility is to be expected in any serum obtained from animals or man injected with solutions

having the complexity of pollen extracts. It is concluded that presently available in vitro methods are ill-suited to the measurement of antibody for skin-reactive components in pollen extracts. These considerations could apply equally well to the measurement by in vitro means of antibody for any biologically active antigen occurring in association with other antigens.

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REFERENCES

1. Prausnitz, C., Kolle, W., and von Wassermann, A.: Handbuch der pathogenen Mikroorganismen, ed. 2, vol. 2, Jena, 1913, Gustav Fischer, chap. XX.
2. Clowes, G. H. A.: A Preliminary Communication on Certain Specific Reactions Exhibited by Hay Fever Cases, Proc. Soc. Exper. Biol. & Med. **10**: 69, 1912-1913.
3. Albus, G.: Beeinflussung der Antikörperkonzentration durch specifische Desensibilisierung bei Pollenallergie, Ztschr. f. d. ges. exper. Med. **95**: 703, 1935.
4. Albus, G.: Serologische Allergenbestimmung bei Pollenkrankheit unter Berücksichtigung des Antikörpertiters, Ztschr. f. d. ges exper. Med. **96**: 710, 1935.
5. Hensel, M. E., and Sheldon, J. M.: Complement Fixation With Specific Allergens in Hay Fever, J. Lab. & Clin. Med. **26**: 1586, 1941.
6. Cavelti, P. A.: Complement Fixation Studies in Allergy, J. ALLERGY **21**: 532, 1950.
7. Portnoy, J., and Sherman, W. B.: Complement Fixation Studies in Ragweed Allergy. II. Determination of Antibody in Human Sera to Ragweed Antigen by Means of a Complement Fixation Inhibition Test; the Relationship of Antibody so Determined to the Passive Transfer for Blocking Antibody, J. ALLERGY **25**: 229, 1954.
8. Feinberg, S. M.: Allergy in Practice, Chicago, 1944, The Year Book Publishers Inc., p. 73.
9. Alexander, H. L., Johnson, M. C., and Bukantz, S. C.: Studies on Correlation Between Symptoms of Ragweed Hay Fever and Titer of Thermostable Antibody, J. ALLERGY **19**: 1, 1948.
10. Cohen, M. B., and Weller, R. R.: Precipitins in the Sera of Patients With Clinical Allergy, J. ALLERGY **12**: 242, 1941.
11. Swineford, O., Jr., and Houlihan, R.: Antibodies to Ragweed Extract; Demonstration by Collodion Particle Technique in Immune Rabbit Serum but not in Serum of Treated Patients, J. ALLERGY **18**: 190, 1947.
12. Lowell, F. C.: Personal communication.
13. Orlans, E. S., Rubenstein, L. J., and Marrack, J. R.: Detection of Antibodies in the Serum of Patients Suffering From Hay Fever, Acta allergol. **6**: 33, 1953.
14. Britton, C. J. C., and Coombs, R. R. A.: The Serological Examination of Sera From Normal Persons and From Hay Fever Patients Both Before and After Specific Hyposensitization for Heatstable Antibodies to the Proteins of Timothy Grass Pollen, Acta allergol. **8**: 31, 1955.
15. Feinberg, R. J., Davison, J. D., and Flick, J. A.: The Detection of Antibodies in Hay Fever Sera by Means of Hemagglutination, J. Immunol. **77**: 279, 1956.
16. Augustin, R.: Standardization of Pollen Extracts, Proc. Brit. A. Allergists **6**: 334, 1953.
17. Coca, A. F.: Studies in Specific Hypersensitiveness. V. The Preparation of Fluid Extracts and Solutions for Use in the Diagnosis and Treatment of the Allergies With Notes on the Collection of Pollens, J. Immunol. **7**: 163, 1922.
18. Cooke, R. A., and Stull, A.: The Preparation and Standardization of Pollen Extracts for the Treatment of Hay Fever, J. ALLERGY **4**: 87, 1933.
19. Stull, A., Sherman, W. B., and Hampton, S. F.: Antigenic Fractions in Ragweed Pollen. I. Water-Soluble Fractions, J. ALLERGY **12**: 117, 1941.
20. Newell, J. M.: Electrophoretic Studies on the Chemical Fractionation of Ragweed Pollen Extracts, J. ALLERGY **14**: 444, 1943.
21. Ouchterlony, O.: Antigen-Antibody Reactions in Gels, Acta path. et microbiol. scandinav. **26**: 507, 1949.
22. Sherman, W. B., and Stull, A.: Antigenic Fractions in Ragweed Pollen. III. Further Evidence of Immunologic Specificity of Fraction 1 and Fraction 2, J. ALLERGY **18**: 287, 1947.
23. Bukantz, S. C., Rein, C. R., and Kent, J. F.: Studies in Complement Fixation. II. Preservation of Sheep's Blood in Citrate Dextrose Mixtures (Modified Alsever's

Solution) for Use in the Complement Fixation Reaction, J. Lab. & Clin. Med. **31**: 394, 1946.

24. Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M.: The Activating Effect of Magnesium and Other Cations on the Hemolytic Function of Complement, J. Exper. Med. **84**: 343, 1946.
25. Kent, J. F., Bukantz, S. C., and Rein, C. R.: Studies in Complement Fixation. I. Spectrophotometric Titration of Complement; Construction of Graphs for Direct Determination of the 50 per cent Hemolytic Unit, J. Immunol. **53**: 37, 1946.
26. Rice, C. E.: Some Factors Influencing the Selection of a Complement-Fixation Method. II. Parallel Use of the Direct and Indirect Technics, J. Immunol. **60**: 11, 1948.
27. Becker, E. L., and Munoz, J.: Multiplicity of Antigens in Ragweed Pollen Extracts as Demonstrated by the Technic of Oudin, Proc. Soc. Exper. Biol. & Med. **72**: 287, 1949.
28. Loveless, M. H.: Immunological Studies of Pollenosis. I. The Presence of Two Antibodies Related to the Same Pollen Antigen in the Serum of Treated Hay-Fever Patients, J. Immunol. **38**: 25, 1940.
29. Gordon, I., and Gnesh, G. M.: The Effect of Relative Concentration on Complement Fixation by Identical Amounts of Antigen and Antibody, Science **107**: 148, 1948.
30. Bukantz, S. C., Johnson, M. C., and Hampton, S.: Quantitative Immunologic Studies With Allergens. II. Deterioration of Ragweed Pollen Extracts as Measured by Precipitation, Neutralization, and Protein Nitrogen Analysis, J. ALLERGY **20**: 20, 1949.
31. Wodehouse, R. P.: Identification of Ragweed Antigens in Gel Diffusion Precipitates, Ann. Allergy **13**: 39, 1955.
32. Sherman, W. B.: Changes in Serological Reactions and Tissue-Sensitivity in Hay-Fever Patients During the Early Months of Treatment, J. Immunol. **40**: 289, 1941.
33. Loveless, M. H., and Cann, J. R.: Distribution of Allergic and "Blocking" Activity in Human Serum Proteins Fractionated by Electrophoresis Convection, Science **117**: 105, 1953.
34. Rice, C. E.: Complement-Fixing Capacity of Mixed Antigen-Antibody Systems, Annual Report, Div. Lab. and Res., New York State Department of Health, 1946, p. 11.
35. Frandsen, V. A., and Samøe-Jensen, T.: On the Ouchterlony Gel Diffusion Technique in Demonstrating the Occurrence of Antibodies in Allergic Disorders, Acta allergol. **8**: 70, 1955.

INCREASED BLOOD HISTAMINE IN THERMAL INTOLERANCE

Report of a Patient With Cryoglobulinemia

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HISTAMINE has become generally recognized as playing some part in allergic phenomena since the work of Dale and Laidlaw[1] in 1910. Its presence has been demonstrated in many tissues of animals and man,[2] where it exists mainly in a bound inactive state. Little free histamine is found normally in the blood or tissues, but anaphylaxis in animals usually is accompanied by a sharp increase of free histamine in the plasma.[3, 4] In man, tissue that is sensitized may contain large amounts of bound histamine, which can be liberated from tissue sections in vitro by addition of the specific antigen[5]; yet, there have been few reports of significant changes in the amount of histamine in the blood during allergic reactions in vivo.[6-8]

When we encountered a patient who showed a striking response to a cold-sensitivity test,[9] we decided to determine whether or not histamine was involved in the reaction.

REPORT OF CASE

A 56-year-old man came to the Mayo Clinic because of progressive intolerance to both heat and cold for more than two years. Quiet air at less than 70° F. or moving air only slightly below body temperature caused pain in the exposed part, generalized urticaria, malaise, nasal stuffiness, and transient asthma. Exertion to the point of sweating caused generalized urticaria and malaise.

The patient presented a striking appearance. The upper half of each ear was deep blue, and repeated ulcerations had made the margins irregular (Fig. 1). The skin of the upper part of the arms, the trunk, and the thighs showed patchy livedo reticularis, which became more vivid and confluent in the air-conditioned examining room.

When an attempt was made to determine the erythrocytic sedimentation rate, it became evident that a cold-precipitable substance was present in the blood, a condition designated as cryoglobulinemia by Lerner and Watson.[10] This substance was present in an amount of 800 mg. per 100 ml. of serum, and it migrated during electrophoresis to the region occupied by gamma globulin. Subsequent studies, including guinea pig sensitization and gel diffusion, showed that the cryoglobulin behaved immunologically much like normal human gamma globulin. Results of studies for multiple myeloma, often associated with cryoglobulinemia, were negative, and tests for lupus erythematosus and cold agglutinins gave normal results. However, there was an absence of serum complement.

A cold-sensitivity test was performed by immersing the hand and an area well above the wrist in water at 10° C. for seven minutes. Almost immediately after the hand was

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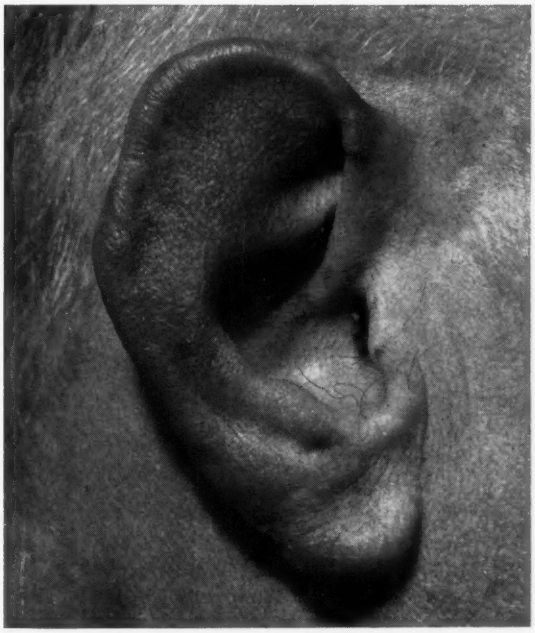

Fig. 1.—Ear showing cyanosis, marginal ulcerations, and loss of tissue from the helix secondary to prior healed ulcerations.

Fig. 2.—Mottling of left hand five minutes after it was removed from immersion in cold water.

taken from the cold water its color changed from pale blue to bright pink; it then became blotchy red and began to itch and swell (Fig. 2). The redness was replaced within five minutes by a waxy yellow-white color with blotchy bluish zones. The swelling became so severe that the hand could not be closed (Fig. 3). The edema remained maximal for several

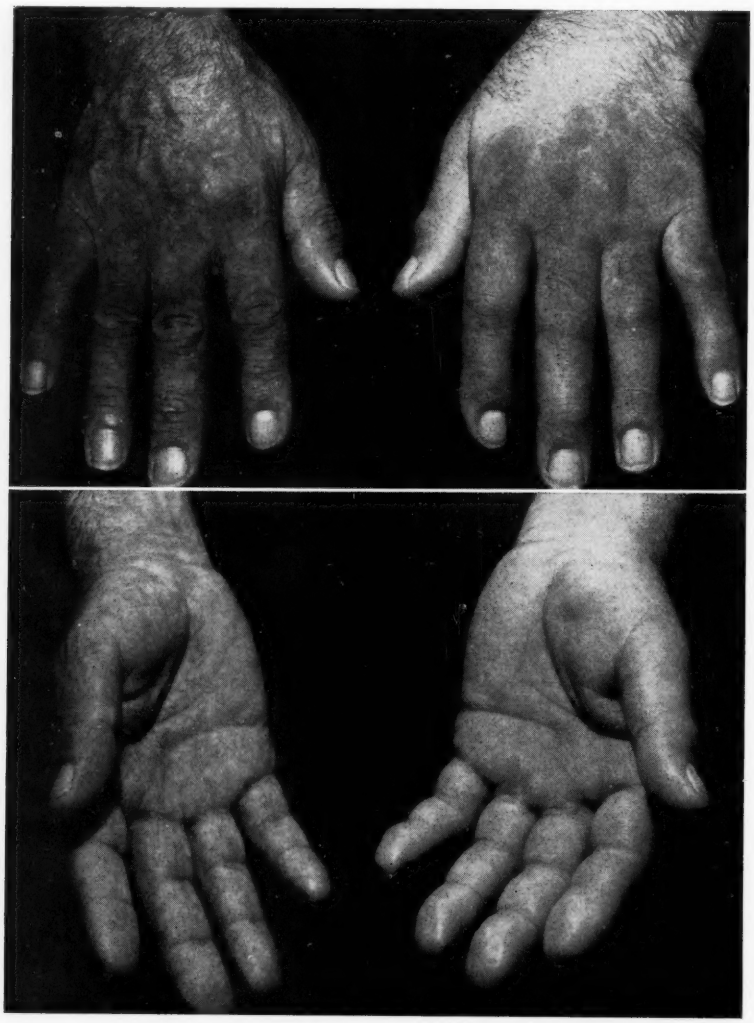

Fig. 3.—Swelling and pallor of left hand twenty-five minutes after it was removed from immersion in cold water.

hours and then gradually subsided during the next two days. Seconds after removal of the tourniquet, the patient felt weak, his face flushed, and he experienced an uncomfortable warmth. We considered that the reaction was typical of severe hypersensitivity to cold and on that basis decided to make the histamine tests.

HISTAMINE STUDIES

Methods.—All samples of blood were drawn from the antecubital vein through a 20-gauge sterile needle into a siliconed syringe. Clotting was pre-

vented by the addition of a sterile solution of heparin, which was used to rinse both the needles and the syringes just before the blood was taken. Samples for determination of the concentration of histamine in whole blood and for enumeration of eosinophils and basophils were removed while the blood was in the syringe. The remainder of the blood was placed in chilled siliconed centrifuge tubes, and the cells and plasma were separated by centrifugation for fifteen minutes at 3,000 to 4,000 r.p.m. Great care was taken to minimize trauma to the cells in order that as few as possible might be disrupted.

The histamine was extracted from the whole blood and plasma according to a modification[11] of the Barsoum-Gaddum procedure,[12] and from the urine by the procedure developed by Roberts and Adam.[13] In all instances the concentration of histamine was determined by assay on an isolated strip of guinea pig ileum.[14]

The eosinophils were enumerated by the direct dilution and counting chamber technique of Randolph,[15] using phloxine stain. The basophils were counted by a similar procedure, with toluidine blue as the stain.[16]

Procedure.—The changes in the concentration of histamine and in the number of eosinophils in the blood during a reaction to cold were determined on three occasions, separated by intervals of eighteen and four months. The procedure followed on the three occasions was almost identical. The patient came to the laboratory without breakfast. The tests were performed while the patient lay in a comfortable supine position. A control sample of blood was taken. A sphygmomanometer cuff that served as a tourniquet was placed on the arm but was left uninflated. The hand and wrist of this arm then were immersed in water at 10° C. for seven minutes. One or two minutes before the hand was withdrawn the cuff was inflated to a pressure well above the patient's systolic blood pressure; the inflation was maintained at this level until the hand was dried and warmed a little bit by holding and rubbing it for three to five minutes. The tourniquet then was released and, as the blood flushed through the hand, a sample was taken. As soon as this was obtained another sample was withdrawn.

An effort was made on one occasion to determine whether any histamine that might have been released in the hand was excreted in the urine. With the patient fasting, the urine was collected during the eleven hours prior to the test and then a separate collection was made during the two hours afterward.

Results.—

Reaction to immersion of hand in cold water: In every test the patient experienced a local and a general reaction. The hand passed through the same changes noted at the original test. The general reaction was first noticeable about three to five minutes after the tourniquet was released, while the hand was still red. A flush appeared over the forehead, face, and thorax. On one occasion the patient became moderately asthmatic and coughed up blood-tinged mucus. The conjunctiva became severely injected and tears flowed; watery rhinitis and nasal congestion resembling severe hay fever were noted. He complained of faintness but remained conscious. At this point during one of the tests

the tourniquet was reinflated and the symptoms rapidly subsided. The pressure then was released and the entire picture reappeared but again subsided with reapplication of pressure. By this time about seven minutes had elapsed since the hand had been removed from the water; when the tourniquet was released for the third time, the general symptoms recurred with less severity and gradually subsided.

After each test the patient felt able to be up after thirty minutes, but for twenty-four hours he felt weak and anorexic and he perspired easily. On one occasion, he had a slight fever from the sixth to the twelfth hour after the test.

Release of histamine during reaction: The concentration of histamine in whole blood coming from the hand was consistently increased by ten to twenty-six times after exposure to cold (Table I). The control samples yielded approximately 2 mcg. of histamine per 100 ml. of blood, which is regarded as the average normal.[17]

Although the concentration of histamine in whole blood increased decisively during the reaction, it was not clear whether the histamine was in the plasma and free there to produce its physiologic effects. The patient's symptoms indicated that this was the case; to settle this point, the concentration of histamine in the plasma was determined. Little, if any, histamine was detectable in the plasma of the control samples of blood (Table I). In contrast, the

TABLE I. CONCENTRATION OF HISTAMINE IN WHOLE BLOOD AND PLASMA FROM HAND BEFORE AND DURING REACTION TO COLD

		HISTAMINE (MCG. PER 100 ML.)					
		BEFORE EXPOSURE TO COLD		MINUTES AFTER EXPOSURE TO COLD			
				3½ TO 5		5+ TO 6	
TEST	DATE	WHOLE BLOOD	PLASMA	WHOLE BLOOD	PLASMA	WHOLE BLOOD	PLASMA
1	December, 1954	2.3	1.2 1.5*	†	†	25.0	33.0 35.0*
2	June, 1956	1.8	<0.5	2.3	2.3	47.6	70.0
3	October, 1956	1.6	0.4	19.7	24.2	22.9	36.0

*Plasma histamine assayed directly, without chemical treatment, on guinea pig ileum.
†No sample taken.

plasma of blood coming from the hand during the reaction contained a high concentration of histamine. When plasma obtained during a reaction was taken directly from the centrifuge tube and applied to guinea pig intestine, it caused a vigorous contraction. In order to assay the strength of this contraction against that produced by histamine, dilution of the plasma was necessary. The quantities estimated to be present in the diluted samples checked well with those obtained by the more refined procedure involving purification of the histamine by extraction (Table I, Test 1).

The following test was made to determine whether chilling the blood liberated histamine from its formed elements into the plasma. Blood was drawn without exposing the hand to cold; it then was separated into two aliquots, one of which was centrifuged warm, while the other was chilled and centrifuged.

The plasma of each yielded extremely small and identical amounts of free histamine, indicating that the cold did not release histamine from the formed elements of the blood.

As a further test, solutions of histamine were made up in concentrations similar to some of the values obtained by assay. Pyrilamine maleate (Neo-Antergan) was added to guinea pig intestine and each prepared histamine solution and its corresponding extract were tested in turn. The reduction of the histamine effect was found to be of about equal magnitude for both members of each pair.

At the time of the last test, we checked the output of free histamine in the urine before and after exposure to cold (Table II). The value was 0.68 mcg. per hour before exposure, which is an average normal, whereas the value after exposure was approximately doubled.

TABLE II. URINARY FREE HISTAMINE BEFORE AND AFTER REACTION OF HAND TO COLD

CONDITION	HOURS OF COLLECTION	HISTAMINE (MCG. EXCRETED PER HOUR)
Before cold	11	0.68
After cold	2	1.31

Changes in eosinophils and basophils in blood from hand: The cells were enumerated in the blood drawn during the first two tests. The pattern of the changes was the same in each (Table III). The number of eosinophils was drastically reduced as the reaction developed, but the number of basophils was not significantly changed.

TABLE III. NUMBER OF EOSINOPHILS AND BASOPHILS IN VENOUS BLOOD FROM HAND DURING REACTION TO COLD

CELLS	TEST	CELLS PER CUBIC MILLIMETER OF BLOOD		
		BEFORE EXPOSURE TO COLD	MINUTES AFTER EXPOSURE TO COLD	
			3½ TO 5	5+ TO 6
Eosinophils	1	101	*	9
	2	86	78	16
Basophils	1	9	*	6
	2	9	11	8

*Not determined.

COMMENT

The local and general reactions of the patient after artificial exposure to cold are explained adequately on the basis of the histamine liberated in the hand and present in the blood returning from the exposed part. Although the entire body was not chilled or warmed during these tests, it appears likely that the reactions resulting from such exposures normally incurred by the patient were mediated by the same process. The increase in urinary histamine is compatible with an increased endogenous turnover of that substance. The cellular changes suggest that the eosinophils participated in the reaction in some way, whereas the basophils did not. The site of participation was not settled by our tests, since we did not know the numbers of eosinophils in the blood entering

the hand while the reaction developed. The contrast in the behavior of these two types of cells is in agreement with other experiences, indicating that the eosinophil is the more labile of the two.[18]

The role of cryoglobulin in this case remains obscure, but it appears unlikely that it is purely coincidental. However, despite the fact that livedo, ulcerations of the ear, and pain on chilling are all commonly seen in cryoglobulinemia, the flushing and the swelling of the hand with subsequent pallor after exposure to cold are typical of simple hypersensitivity to cold. Furthermore, hypersensitivity to heat, which our patient also manifested, has not been mentioned in any of the reported cases of cryoglobulinemia coming to our attention. That the increase in blood histamine is not a necessary accompaniment of cryoglobulinemia was demonstrated in another of our patients, whose serum contained 1 Gm. of cold-precipitable substance per 100 ml. of serum. After this patient was exposed under the conditions just outlined, we found no increase in blood histamine and no clinical manifestations of simple hypersensitivity to cold.

It is possible that cryoglobulinemia could potentiate the physical allergy by slowing the peripheral circulation or by actual vascular damage. The further possibility exists that cryoglobulin participates in a massive antigen-antibody reaction, with consequent release of histamine. While this concept is intriguing, there is no conclusive evidence as to whether the substance would be acting as an antigen or an antibody, or whether it even represents an antigen-antibody complex, as variously suggested in the literature.

SUMMARY

A 56-year-old man had progressive intolerance to both heat and cold. When his hand was withdrawn from cold water, the patient experienced a local and a general reaction, and greatly increased quantities of free histamine were found in the plasma of the blood leaving the chilled extremity. The patient also had a large amount of cryoglobulin in his serum. The relationship of this concomitant cryoglobulinemia to the other findings is not known.

REFERENCES

1. Dale, H. H., and Laidlaw, P. P.: The Physiological Action of B-Iminazolylethylamine, J. Physiol. **41:** 318, 1910.
2. Feldberg, W.: Distribution of Histamine in the Body. In Ciba Symposium on Histamine, Boston, 1956, Little, Brown & Company, pp. 4-13.
3. Code, C. F.: The Histamine Content of the Blood of Guinea Pigs and Dogs During Anaphylactic Shock, Am. J. Physiol. **127:** 78, 1939.
4. Dragstedt, C. A.: Anaphylaxis, Physiol. Rev. **21:** 563, 1941.
5. Schild, H. O.: Histamine Release and Anaphylaxis. In Ciba Symposium on Histamine, Boston, 1956, Little, Brown & Company, pp. 139-149.
6. Rose, Bram: Studies on Blood Histamine in Cases of Allergy: Blood Histamine During Wheal Formation, J. ALLERGY **12:** 327, 1941; correction **12:** 522, 1941.
7. Rose, Bram: Histamine, Hormones and Hypersensitivity. In Pincus, G. (editor): Recent Progress in Hormone Research, New York, 1952, Academic Press, Inc., vol. 7, pp. 375-398.
8. Capps, R. B., and Young, R. H.: Hypersensitivity to Light: Studies of an Unusual Case Treated Successfully With Histamine, J. Clin. Invest. **19:** 778, 1940.
9. Horton, B. T., Brown, G. E., and Roth, Grace M.: Hypersensitiveness to Cold With Local and Systemic Manifestations of a Histamine-like Character: Its Amenability to Treatment, J. A. M. A. **107:** 1263, 1936.

10. Lerner, A. B., and Watson, C. J.: Studies of Cryoglobulins: Unusual Purpura Associated With Presence of High Concentration of Cryoglobulin (Cold Precipitable Serum Globulin), Am. J. M. Sc. **214**: 410, 1947.
11. Code, C. F.: Quantitative Estimation of Histamine in Blood, J. Physiol. **89**: 257, 1937.
12. Barsoum, G. S., and Gaddum, J. H.: The Pharmacological Estimation of Adenosine and Histamine in the Blood, J. Physiol. **85**: 1, 1935.
13. Roberts, M., and Adam, H. M.: New Methods for Quantitative Estimation of Free and Conjugated Histamine in Body Fluids, Brit. J. Pharmacol. **5**: 526, 1950.
14. Code, C. F., and McIntire, F. C.: The Quantitative Determination of Histamine. In Methods of Biochemical Analysis, New York, 1956, Interscience Publishers, Inc., vol. 3, pp. 49-95.
15. Randolph, T. G.: Blood Studies in Allergy: Direct Counting Chamber Determination of Eosinophils by Propylene Glycol Aqueous Stains, J. ALLERGY **15**: 89, 1944.
16. Moore, J. E., III, and James, G. W., III: Simple Direct Method for Absolute Basophil Leucocyte Count, Proc. Soc. Exper. Biol. & Med. **82**: 601, 1953.
17. Code, C. F.: Histamine in Blood, Physiol. Rev. **32**: 47, 1952.
18. Code, C. F., and Mitchell, R. G.: Histamine, Eosinophils, and Basophils in the Blood, J. Physiol. **136**: 449, 1957.

SENSITIVITY TO ANIMALS: A CASE REPORT WITH IMMUNOLOGIC STUDIES

I. Clinical Aspects

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THIS article concerns the exquisite sensitivity of a patient to mouse dander and other animal epidermals. His marked positive direct skin reactions to very high dilutions of various animal danders, the broad spectrum of animal sera sensitivities, the positive Prausnitz-Küstner reactions, and the unusual finding of hemagglutinating antibodies in the serum of this patient will be described.

Animal epidermals as a cause of clinical allergy are well known. The most common animal danders that cause allergic symptoms are those of the domestic animals, such as the cat, the dog, the horse, and the chicken. Rabbit hair, wool, and cow epidermal also are very frequently offenders. Guinea pigs, rats, mice, etc., have occasionally been the causative factors of allergic reactions in laboratory workers. Gilday[1] recently reported a case of bronchial asthma caused by rat hair.

A close correlation between dander and serum sensitivity was demonstrated by Ratner and Gruehl, Tuft, Forster, and others.[2-5] The dangers of administering horse serum to patients who are sensitive to horse dander have been observed for many years. Silver and Bookman[6] indicated that cattle dander and horse dander resemble, in molecular weight and electrophoretic migration, the albumin fraction of their respective sera. Accordingly, the present report encompasses sensitivity to animal sera, as well as to the corresponding epidermal extracts, as significant antigens for this patient.

Simon[7] reported the relationship of sensitivities to horse dander and to horse serum. He noted that several patients who had positive scratch test reactions to horse serum also showed positive skin tests to many other mammalian sera.

This first report will cover clinical aspects, studies on direct skin tests, quantitative and qualitative passive transfer tests, and neutralization tests. A report of the serologic studies (hemagglutination) of this serum will be presented in a subsequent article.

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CASE REPORT

G. A. U., a 29-year-old man, a graduate student in physiology, contacted us for the first time on March 27, 1956. That day, while working with white mice, he was scratched on the arm and within a few minutes developed a markedly edematous forearm and severe asthma.

He had a past history of allergic rhinitis and bronchial asthma; the former condition had been present for fifteen years and the latter for eight years. When he was a child, exposure to guinea pigs, white rats, and dogs was known to produce allergic symptoms. In 1947 an allergy survey revealed positive reactions to house dust, fungi, and insects.* A positive test reaction to rat dander extract was noted in 1951.

The patient had worked with white mice from 1951 to 1953 without difficulty. Upon re-exposure in 1956, however, he developed his present complaints of rhinitis and asthma whenever he entered the animal room housing the white mice. In view of this severe clinical sensitivity, it was deemed advisable to investigate the patient's hypersensitivity very thoroughly but with extreme caution.

MATERIALS

1. Dander extracts were prepared by defatting the hair and epidermal scales with toluol and then extracting the residue for five days with enough phosphate-buffered saline, pH 7.0, to make a thick paste. The liquor was then filtered, dialyzed against the extracting fluid, and finally passed through a Seitz filter. Kjeldahl determinations were made to find the phosphotungstic acid nitrogen (PTAN) content of each extract.

2. Sera from the more common laboratory animals and from the domestic animals were readily obtainable; sera from foreign and wild species were obtained from the Rutgers Serological Museum through the courtesy of Dr. Alan Boyden.

All dander extracts and animal sera were passed through Seitz filters and tested for sterility before use.

METHODS AND RESULTS

Direct Skin Tests.—Direct intradermal skin tests were performed with various animal danders and sera, including mouse, rat, horse, dog, and cat danders and sera, by first using dilutions of 1:1,000,000 and 1:100,000 of the freshly prepared solutions.

As shown in Table I, these tests gave unequivocally positive results with most materials used. In many instances, tests with both dander extract and serum in high dilutions were strongly positive.

For example, mouse dander and serum produced the strongest reactions of all danders and sera tested. Even a dilution of 1:100,000,000 of mouse serum elicited a definite positive intradermal skin test reaction; the reaction to mouse dander was positive in a dilution of 1:10,000,000 but there was no reaction to a dilution of 1:100,000,000.

In some instances, extracts and sera were not sufficiently diluted to permit us to ascertain the minimal amount needed to produce a positive reaction. These

*Information supplied by Dr. Samuel M. Feinberg, Chicago, Illinois.

tests included antigens with which there had been known contact and which had precipitated allergic symptoms. Because of the almost uniformly positive results with all of the substances tested, except for cat extract, these tests were extended to include materials with which there was no known or likely contact.

Accordingly, sera from foreign undomesticated animals were tried, and positive results were obtained with lion, coyote, polar bear, Indian elephant, and tiger sera. Coyote and polar bear sera in dilutions to 1:100,000 elicited positive skin reactions, whereas the reaction to tiger serum was positive only in a dilution up to 1:1,000.

TABLE I. DIRECT SKIN TESTS ON G. A. U.

ANIMAL	DANDER EXTRACT DILUTION	REACTION	SERUM DILUTION	REACTION
1. Mouse	1:10,000,000*	++	1:100,000,000*	++
2. Rat	1:100,000	++	1:10	++++
3. Guinea pig	1:10,000	+++	1:100,000	++++
4. Horse	1:10	++++	1:100,000	++
5. Beef	1:10	+	1:1,000	++++
6. Cat hair	1:10	0	1:1,000	++++
7. Rabbit	1:10	+		
8. Sheep wool	1:10	+++		
9. Feathers	1:10	+		
10. Hog			1:100,000	++++
11. Lion			1:10	++++
12. Coyote			1:100,000*	++
13. Elephant			1:10	+
14. Polar bear			1:100,000*	++
15. Tiger			1:1,000*	++

Key to symbols:

+ = A small area of erythema.
++ = Erythema and small wheal.
+++ = A larger area of wheal and erythema.
++++ = Erythema and wheal formation with pseudopods.
*Highest dilution which produced definite positive skin reaction.

Passive Transfer Tests.—The patient's serum was separated sterilely and was kept frozen until needed. Dilutions of it were made, using buffered saline (pH 7.0).

Recipients for passive transfer studies included twelve healthy nonallergic male medical students, three male allergy patients, and one female allergy patient. The recipients had given negative responses to the antigen being used. In the preliminary qualitative tests, intradermal injections of 0.05 ml. undiluted or 1:4 diluted patient's serum were made in order to sensitize several sites on the outer aspects of the recipients' upper arms, and the sites were tested intradermally with 0.01 ml. of the various antigens twenty-four hours later.

Quantitative tests were carried out in the same manner except that the backs, rather than the arms, of the recipients were used and further dilutions of the serum were injected in order to determine a titer of the serum when tested with a constant dilution of antigen in all sites. In all passive transfer studies one sensitized site was injected with saline rather than antigen when the tests were performed, and one site was injected with saline rather than the sensitizing serum when the sites were prepared. These sites served as controls.

To determine specificity of the antibody involved, repeated injections of the previously prepared sites with the same and different antigens (in vivo neutralization) were made at twenty-four-hour intervals.

All passive transfer tests were read twenty minutes after testing with the antigen. An area of erythema was taken as a one-plus reaction. An area of erythema and a small wheal was read as two-plus. A larger reaction was called a three-plus. Erythema and wheal with the appearance of pseudopods was read as four-plus.

Sites prepared with concentrated G. A. U. serum and tested with mouse, beef, hog, horse, and guinea pig sera diluted 1:10 gave four-plus reactions (Table II, column A). Cat serum 1:10 did not precipitate a reaction. Repeat testing of the sites with homologous sera gave negative reactions except in the case of mouse serum, which gave a positive but much weaker response the second time, indicating partial neutralization of the antibody (Table II, column B).

TABLE II. QUALITATIVE PASSIVE TRANSFER TESTS AND ANTIBODY EXHAUSTION STUDY

SITE	A 10/16/56			B 10/18/56			C 10/19/56		
1	Mouse serum	1:10	++++	Mouse serum	1:10	++	Rat	1:10	0
2	Beer serum	1:10	++++	Beef serum	1:10	0	Rat	1:10	++++
3	Hog serum	1:10	++++	Hog serum	1:10	0	Rat	1:10	++++
4	Horse serum	1:10	++++	Horse serum	1:10	0	Rat	1:10	++++
5	Guinea pig	1:10	++++	Guinea pig	1:10	0	Rat	1:10	++++
6	Cat serum	1:10	0	Cat serum	1:10	0	Rat	1:10	++++
7	Control (saline)		0	Mouse serum	1:10	++++	Rat	1:10	0

Sites were prepared on Oct. 15, 1956, with 0.05 ml. concentrated G. A. U. serum. Recipient was J. O. K. I.

Testing each site a third time with rat serum elicited four-plus reactions except in instances where sites had previously been injected with mouse serum (Table II, column C). As shown in Table III, sites prepared in three recipients with G. A. U. serum diluted 1:4 reacted strongly when tested with lion, polar bear, and coyote sera. Negative tests were noted in sites tested with tiger and Indian elephant sera.

TABLE III. QUALITATIVE PASSIVE TRANSFER TEST

ANTIGEN	RECIPIENT F. I. N.	Y. A. R.	K. U. P.
1. Lion serum 1:10	++++	++++	++++
2. Tiger serum 1:10	0	0	0
3. Elephant serum 1:10	0	0	0
4. Polar bear serum 1:10	+++	+++	+
5. Coyote 1:10	++++	++++	++
6. Mouse serum 1:100	++++	++++	++++
7. Saline control	0	0	0

Sites sensitized twenty-four hours prior to testing with G. A. U. serum 1:4, 0.05 ml.

Quantitative passive transfer studies, as presented in Table IV-A, revealed positive reactions when mouse serum 1:100 was used to test sites previously prepared with G. A. U. serum diluted up to 1:64. Dog serum and guinea pig

serum produced positive reactions in the antibody (G. A. U. serum) in dilutions of 1:8 and 1:16, respectively, whereas bovine and rabbit sera failed to cause reactions in this test.

Twenty-four hours after these initial tests the sites that were previously tested with mouse serum 1:100 were again injected with the same dilution of

TABLE IV-A. QUANTITATIVE PASSIVE TRANSFER STUDY

		ANIMAL SERA (ANTIGENS)*				
SITE	DILUTION	A MOUSE 1:100	B DOG 1:10	C BOVINE 1:10	D GUINEA PIG 1:10	E RABBIT 1:10
1	1:2	++++	++++	–	++++	–
2	1:4	++++	+++	–	++++	–
3	1:8	++++	+	–	+	–
4	1:16	++++	–	–	++	–
5	1:32	++++	–	–	–	–
6	1:64	+	–	–	–	–
7	1:128	–	–	–	–	–
8	1:256	–	–	–	–	–
9	Saline	–	–	–	–	–

Recipient: F. L. O.
Sites sensitized with 0.05 ml. G. A. U. serum diluted with saline.
*Sites tested twenty-four hours after sensitization.

TABLE IV-B. QUANTITATIVE PASSIVE TRANSFER STUDY

		ANIMAL SERA (ANTIGENS)*				
SITE	DILUTION	A MOUSE 1:100	B DOG 1:10	C MOUSE 1:100	D GUINEA PIG 1:10	E HOG 1:10
1	1:2	+	–	++++	+	–
2	1:4	+	–	++++	–	–
3	1:8	–	–	+++	–	–
4	1:16	–	–	+++	–	–
5	1:32	–	–	+	–	–
6	1:64	–	–	–	–	–
7	1:28	–	–	–	–	–
8	Saline	–	–	–	–	–

Recipient: F. L. O.
Sites sensitized with 0.05 ml. G. A. U. serum diluted with saline.
*Sites tested twenty-four hours after initial testing (Table IV-A) and forty-eight hours after sensitization with G. A. U. serum dilutions.

TABLE IV-C. QUANTITATIVE PASSIVE TRANSFER STUDY

		ANIMAL SERA (ANTIGENS)*				
SITE	DILUTION	A DOG 1:10	B MOUSE 1:100	C GUINEA PIG 1:10	D†	E†
1	1:2	–	++++	–		
2	1:4	–	++++	+		
3	1:8	–	+++	–		
4	1:16	–	+++	–		
5	1:32	–	+	–		
6	1:64	–	–	–		
7	1:128	–	–	–		

Recipient: F. L. O.
Sites sensitized with 0.05 ml. G. A. U. serum diluted with saline.
*Sites tested seventy-two hours after sensitization.
†Not retested.

mouse serum. Minimally positive reactions in the first two sites—G. A. U. 1:2 and 1:4—were obtained (Table IV-B, column A). Retests of the sites previously tested with dog serum by the homologous serum antigen gave entirely negative reactions (column B), indicating complete neutralization in vivo of the passively transferred antibody.

Mouse serum reacted in the sites previously tested with bovine serum, giving only slightly diminished reactions as compared with sites initially tested with mouse serum (column C). Guinea pig serum used to test sites previously tested with guinea pig serum yielded a slightly positive reaction in the first dilution of G. A. U. serum (column D). Hog serum 1:10 gave entirely negative responses in sites previously negative when tested with rabbit serum 1:10 (column E).

TABLE IV-D. QUANTITATIVE PASSIVE TRANSFER STUDY

		ANIMAL SERA (ANTIGENS)*	
		A GUINEA PIG 1:10	B DOG 1:10
SITE	DILUTION		
1	1:2	+	+
2	1:4	+	+
3	1:8	–	–
4	1:16	–	–
5	1:32	–	–
6	1:64	–	–

Recipient: F. L. O.
Sites sensitized with 0.05 ml. G. A. U. serum diluted with saline.
*Sites tested ninety-six hours after sensitization.

On the following day sites twice previously tested with mouse serum were injected with dog serum and gave uniformly negative results (Table IV-C). On the other hand, mouse serum injected into sites twice previously tested with dog serum elicited positive reactions similar to those obtained earlier on initial testing with mouse serum. Guinea pig serum in sites tested once with bovine serum and then with mouse serum produced a slightly positive reaction in the site prepared with G. A. U. serum 1:4 only; the 1:2 site was negative, presumably because of a technical error.

On further retesting (Table IV-D) with guinea pig serum in sites previously tested with dog serum 1:10 and then once with mouse serum 1:100, one-plus reactions in the lowest dilutions of G. A. U. serum developed. Dog serum in sites tested twice with guinea pig serum 1:10 also gave one-plus reactions in lower dilutions of the patient's serum (Table IV-D).

In Vitro Neutralization Tests.—In vitro neutralization studies were carried out by incubating equal volumes of varying dilutions of animal serum (antigen) and threefold diluted G. A. U. serum (reagin) for twenty-four hours at 4° C. Approximately 0.05 ml. of each of these mixtures was then injected intradermally in various areas in normal recipients. All of the sites were marked for identification and then were tested twenty-four hours later with mouse serum diluted 1:100.

As shown in Table V, antimouse-serum antibody in G. A. U. serum 1:3 was completely neutralized by mouse serum diluted to 1:10,000. A site prepared with G. A. U. serum 1:3 incubated with mouse serum diluted to 1:20,000 or higher gave four-plus reactions when tested with mouse serum 1:100. As a control, saline incubated with G. A. U. serum 1:3 and used to prepare a site produced a three-plus reaction when tested. No evidence of any precipitate was noted in any of the tubes in which the antiserum-antigen mixtures were incubated.

TABLE V. IN VITRO NEUTRALIZATION STUDY OF ANTIBODY IN G. A. U. SERUM BY MOUSE SERUM

SITE	MOUSE SERUM DILUTIONS (0.2 ml.)*	REACTION (TESTED TWENTY-FOUR HOURS LATER WITH MOUSE SERUM DILUTED 1:100)
1	1:10	–
2	1:100	–
3	1:1,000	–
4	1:5,000	–
5	1:10,000	–
6	1:20,000	++++
7	1:40,000	++++
8	1:100,000	+++
9	1:500,000	++++
10	Saline	+++

Recipient: K. A. R.

*Varying dilutions of mouse serum (0.2 ml.) mixed with an equal volume of 1:3 diluted G. A. U. serum and kept overnight at 4° C.

The heat lability of the antibody involved was determined by sensitizing sites with varying dilutions of unheated serum (G. A. U.) and a like series of dilutions of this same serum that had been heated for two hours at 56° C. Both sets of sites were challenged twenty-four hours later with mouse serum diluted 1:100.

TABLE VI. EFFECT OF HEATING SERUM AT 56° C. FOR TWO HOURS

DILUTIONS OF SERUM	TESTED WITH MOUSE SERUM 1:100	
	HEATED	NOT HEATED
1:2	–	++++
1:4	–	+
1:8	–	+++
1:16	–	+++
1:32	–	+
Saline (control)	–	–

The antibody responsible for passive transfer of sensitivity to mouse serum was found to be heat labile and was completely destroyed after heating at 56° C. for thirty minutes as well as for two hours (Table VI).

DISCUSSION

The presence of heat-labile antibody capable of passive transfer (reagin) is clearly demonstrated in this allergic patient. The numerous reactions to animal products, both sera and danders, apparently resulted from the patient's inherent susceptibility to sensitization and his intimate contact with a variety of animals in the course of his work in biology.

Judging by the results of direct skin tests and passive transfer study, there was by no means equal sensitivity to all of the species tested. Mouse and rat sera gave by far the strongest reactions. Dog and guinea pig sera were also important antigens. Reactions to hog, beef, and rabbit sera were only weakly positive and were negative in quantitative passive transfer studies.

To determine the specificity of the reactions observed, repeated injections of the passively sensitized sites with the same and different antigens were performed. The passive transfer tests were positive with many animal sera; all were negative on retest with the homologous sera, except for mouse serum which gave a positive but considerably reduced response. It is apparent that the passive transfer antibody is effectively neutralized by the particular homologous serum. Rat serum elicited strongly positive reactions from all these sites except those previously tested with mouse serum. Likewise, sites previously exhausted by repeated injections of mouse serum failed to react to the first injection of dog serum. Contrariwise, when sites were previously exhausted by repeated injections of dog serum, positive reactions were obtained when the sites were subsequently tested with mouse serum.

These results would indicate that mouse serum contains antigenic components capable of neutralizing the reactions of G. A. U. serum with heterologous dog and rat sera as well as with the homologous serum. On the other hand, dog, rat, and the other sera are apparently capable of neutralizing only the homologous reactions. Thus, the primary role of mouse sensitivity becomes apparent.

The ability of mouse serum to cross-neutralize dog and rat sera reactions suggests an explanation of the positive reactions obtained with the sera of the wild beasts—lion, polar bear, and coyote. These reactions to species to which the patient had not been directly exposed may represent cross-reactions with antigens in mouse serum.

These findings are in agreement with the reports of Simon,[7, 8] who demonstrated by an in vitro reagin neutralization technique that there were some, but not complete, cross-reactions among the various animal sera.

Inhalant allergens seem to be of prime importance in this case. No foods of animal origin caused symptoms, and no gastrointestinal symptoms were reported. The reactions, on direct skin testing, to the sera and the corresponding epithelial extract were essentially parallel and differed only in intensity. The different degree of reaction may well be due only to the lower protein content of the dander extract. Tests with cat dander extract produced negative reactions, but cat serum produced positive reactions.

Desensitization of the patient with mouse dander in very high dilutions was tried clinically. However, the recurrence of symptoms after very brief contact with dogs again points to the intense nature of his sensitivity.

The patient was advised to discontinue work in his field of study. Since the range of sensitivity was so large, desensitization to each particular species was considered too hazardous and impractical.

SUMMARY AND CONCLUSIONS

1. This report concerns a patient markedly sensitive to mouse, dog, guinea pig, and other animal danders.

2. A close correlation exists between sensitivity to dander and to serum of the same animal, as evidenced by direct skin tests.

3. This patient's serum contains an antibody that has the characteristics of reagin—it was heat labile at 56° C. and was capable of passive transfer to the skin of a nonallergic recipient. It was neutralized by in vitro incubation with a high dilution of the homologous antigen.

4. In qualitative and quantitative passive transfer tests, distinct species specific antibodies directed against most of the species to which the patient is strongly sensitive can be demonstrated in the patient's serum.

5. Mouse serum alone was capable of cross-neutralizing to the other animal sera tested in passive transfer experiments.

6. It is likely that antibodies against one or more antigens can be found widely, but not universally, distributed among mammals. This would account for the patient's sensitivity to some animals with which he has not had direct contact.

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REFERENCES

1. Gilday, F. J.: Bronchial Asthma Due to Rat Hair, Delaware M. J. **28**: 110, 1956.
2. Ratner, B., and Gruehl, H. L.: Anaphylactic Crossed Relationship Between Horse Dander and Horse Serum, Arch. Path. **8**: 635, 1929.
3. Tuft, L.: Further Studies in Serum Allergy. VI. Antigenic Relationship Between Horse Dander and Horse Serum Sensitivity, J. ALLERGY **6**: 25, 1934.
4. Forster, G. F.: Is There an Immunologic Relationship Between Horse Serum and Horse Dander? J. Exper. Med. **47**: 903, 1928.
5. De Besche, A.: Studies on the Reaction of Asthmatics and Passive Transference of Hypersusceptibility, Am. J. M. Sc. **166**: 265, 1923.
6. Silver, D. Z., and Bookman, R.: Studies on the Nature of Allergenic Material in Animal Danders and Pollens, J. ALLERGY **27**: 127, 1956.
7. Simon, F. A.: Allergic Skin Reactions to Mammalian Sera, J. ALLERGY **12**: 610, 1941.
8. Simon, F. A.: Human Allergy to Mammalian Sera, J. Exper. Med. **75**: 315, 1942.

SENSITIVITY TO ANIMALS: A CASE REPORT WITH IMMUNOLOGIC STUDIES

II. Serologic Aspects

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THE serum of a patient who showed extreme clinical hypersensitivity to animal epithelia was studied in our laboratory. Intradermal skin tests and passive transfer studies were strongly positive for many animal dander extracts and animal sera.[1] Since reagins in high titer were found in this serum, efforts were made to demonstrate an antibody reaction in vitro. The presence of antibodies in the serum of allergic persons is not usually demonstrated by direct in vitro serologic techniques. Recently, however, certain investigators[2-5] have shown that in the sera of some pollen-sensitive patients there is a substance which agglutinates red blood cells when coated with the particular pollen extract. In view of these findings, hemagglutination techniques previously developed by Pressman and associates[6] and by Boyden[7] were employed in an effort to demonstrate "antibodies" against animal dander in this patient's serum. A substance causing agglutination of red blood cells coated with antigen was found in this serum.

METHODS AND MATERIALS

Antigens.—Antigens consisted of aqueous animal dander extracts and sera from various animals, including those which had elicited positive intradermal skin test reactions. All of these animal sera were inactivated for thirty minutes at 56° C.

Dander extracts were prepared by defatting the hair and epidermal scales with toluol and then extracting the residue for five days with enough phosphate-buffered saline, pH 7.0, to make a thick paste. The liquor was then filtered, dialyzed against the extracting fluid, and finally passed through a Seitz filter. Macro Kjeldahls were made to determine the phosphotungstic acid nitrogen (PTAN) content of each extract.

Saline tissue extracts of beef, hog, monkey, and human kidney were used. To prepare these extracts, tissue was finely minced and an equal volume of cold saline solution was added. The tissue was extracted by shaking overnight in the refrigerator, producing a 50 per cent extract.

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Serologic Methods.—Human group O Rh negative red blood cells (R.B.C.) were taken in standard ACD solution.* For each day's test the cells were washed three times in phosphate-buffered saline, pH 7.2, and were resuspended in this phosphate buffer to make a 4 per cent suspension. In preparing the phosphate-buffered saline, we used M/15 disodium acid phosphate and M/15 potassium dihydrogen phosphate with an equal volume of 0.9 per cent sodium chloride to adjust the tonicity.

Antiserum was obtained from the patient (G.A.U.) and frozen until ready for use. This serum was inactivated at 56° C. for thirty minutes when used only with the tanned red blood cell method. (This was done in order to prevent hemolysis.) Serial dilutions of antiserum (G.A.U.) were carried out, with normal human serum diluted 1:100 (NHS 1/100) with phosphate-buffered saline used as the diluent. The normal human serum used as the diluent had been inactivated for thirty minutes at 56° C. Final volumes of each dilution in the serial titrations of antiserum (G.A.U.) were 0.1 ml.

Two types of hemagglutination studies were employed: the bis-diazotized benzidine (B.D.B.) method of attaching antigen and the tanned red cell method.

Bis-Diazotized Benzidine (B.D.B.) Method: The procedure was carried out entirely in an ice bath. Bis-diazotized benzidine was freshly prepared by dissolving 1 gram of benzidine hydrochloride in 50 ml. of distilled water and then adding 12 ml. of 1 N hydrochloric acid. Sodium nitrite, 0.8 gram, dissolved in 10 ml. of distilled water, was added slowly with constant stirring until one drop of the mixture gave a positive test with starch-potassium iodide paper. The solution was diluted to 100 ml. with chilled distilled water. A 5 ml. aliquot of this solution was adjusted to pH 7.0, using approximately 6 ml. of 0.1 N sodium hydroxide. One milliliter of the neutralized B.D.B. was then added to 19 ml. of boric acid-sodium borate buffer, pH 8.4.

For linking the R.B.C. with antigen, 2 ml. of the 4 per cent R.B.C. suspension was mixed with an equal volume of suitably diluted antigen solution. (Preliminary experiments indicated that dander extracts provided optimal results when used undiluted, whereas animal sera used as antigens gave better results if diluted 1:10.) To the mixture of R.B.C. and antigen, 1 ml. of B.D.B. was added. It was necessary to combine the red cells and the antigen prior to adding the B.D.B.

The solutions remained in contact for five minutes at room temperature and then were centrifuged at 4° C. for five minutes at 1,200 r.p.m. in an International refrigerated centrifuge, Model PR-2, with head #284. The cells were washed twice in phosphate-buffered saline, pH 7.2, and then twice in NHS 1/200, and finally they were resuspended in NHS 1/200 to a final volume of 5 ml. These antigen-linked red blood cells (volume 0.1 ml.) were then added to each of the serial dilutions of the antiserum. As controls, untreated R.B.C. were added to one tube containing the initial antiserum dilution.

*Acid citrate dextrose (Baxter).

The tubes were then shaken thoroughly and the cells were allowed to settle undisturbed at room temperature. Patterns of the sedimented cells were read after two hours. Strongly agglutinated tests appeared as a round plaque on the bottom of the tube which folded only after tilting of the rack. These were read as four-plus. A more widely dispersed pattern which appeared less tightly agglutinated and which fragmented in coarse clumps on tilting was considered a three-plus reaction. Reactions which were diffusely spread over the entire bottom of the tube with some folding of the edges and which broke into less coarse clumps on tilting were recorded as two-plus. A diffuse pattern with increased granularity which settled unevenly on tilting was interpreted to be a one-plus reaction. Round buttons and doughnutlike rings which streamed down on tilting, as fine sand might, were read as negative.

Tanned Cell Hemagglutination Method: Tannic acid solution was freshly prepared each week by dissolving 0.5 gram of the powdered tannic acid (Baker) in 100 ml. of distilled water. To an equal volume of a 4 per cent suspension of R.B.C. was added a 1:25,000 dilution of tannic acid in buffered saline. This mixture was incubated at room temperature for thirty minutes. These tanned cells were then washed three times with buffered saline. Animal sera, diluted 1:200 with buffered saline, were used as the coating antigens. Equal volumes of diluted antigen and of tanned washed R.B.C. (now a 2 per cent suspension) were combined and allowed to stand at room temperature for thirty minutes. The tanned, coated R.B.C. (now a 1 per cent suspension) were then washed three times with NHS 1/100 and added in 0.1 ml. volumes to an equal volume of antiserum in each of the previously prepared serial dilutions. Washed, tanned, uncoated red blood cells, 0.1 ml., were added as a control to one tube containing an initial dilution of the antiserum.

The tubes were shaken and the cells were allowed to sediment at room temperature for two hours. Patterns were read as in the B.D.B. method, but without tilting, and then the tubes were shaken and kept at 4° C. for eighteen hours and were read again.

RESULTS

Direct Tests.—Mouse dander extract, undiluted, was azo-linked to erythrocytes by the B.D.B. method described previously. Hemagglutination by the patient's serum (G.A.U.) was consistently obtained in dilutions up to 1:9 (Table I). A normal human serum (NHS), included as a control, provided negative results, as did the human erythrocytes not linked to dander antigen.

TABLE I. HEMAGGLUTINATION OF R.B.C. AZO-LINKED TO MOUSE DANDER EXTRACT BY G.A.U. SERUM

DILUTIONS OF HUMAN SERA	G.A.U. (PATIENT)	NORMAL (CONTROL)
1. 1 : 3	+	–
2. 1 : 9	+	–
3. 1 : 27	–	–
4. Saline	–	–
5. 1 : 3 and uncoated R.B.C. (control)	–	–

See text for method of reading hemagglutination tests.

The basis of the B.D.B. test rests on the formation of diazo linkages between the protein of the antigen and the red cell. Protein content of the antigenic solution is a critical factor in obtaining satisfactory results. Serum of the particular species has a much higher protein content than the animal dander extract and may contain an antigen identical with or similar to that responsible for the agglutination of dander-coated R.B.C.[1] Accordingly, tests using mouse serum, instead of mouse dander, as the antigen linked to the red cells were carried out. A 1:10 dilution of mouse serum azo-linked to red cells proved to be optimal and resulted in agglutination of these cells by the patient's serum in dilutions up to 1:320 (Table II).

TABLE II. HEMAGGLUTINATION OF R.B.C. AZO-LINKED TO MOUSE SERUM (1:10) BY G.A.U. SERUM AND EFFECT OF HEATING PATIENT'S SERUM

DILUTION OF HUMAN SERUM	G.A.U. SERUM (PATIENT)		NORMAL SERUM (CONTROL)
	UNHEATED	HEATED AT 56° C. FOR 2 HRS.	
1. 1 : 5	++++	++++	–
2. 1 : 10	++++	++++	–
3. 1 : 20	++++	++++	–
4. 1 : 40	++++	++++	–
5. 1 : 80	++++	+++	–
6. 1 : 160	++	+	–
7. 1 : 320	+	+	–
8. 1 : 640	–	–	–
9. Saline	–	–	–
10. 1 : 5 and washed R.B.C.	–	–	–

When G.A.U. serum was heated at 56° C. for two hours, the hemagglutination titer was not affected (Table II). In contrast, the passive transfer antibody (reagin) was destroyed by this process, as described in the preceding article.[1]

TABLE III. HEMAGGLUTINATION OF R.B.C. AZO-LINKED TO ANIMAL DANDER EXTRACTS (BY G.A.U. SERUM)

ANIMAL DANDERS	TITER* OF G.A.U. SERUM
1. Mouse	9
2. Rat	20
3. Horse	80
4. Cat	32
5. Hog	32
6. Sheep	32
7. Rabbit	32
8. Dog	0
9. Beef	0

*Reciprocal of highest dilution of patient's serum producing agglutination of R.B.C. coated with various animal dander extracts.

The presence of antibody to mouse serum and mouse dander, as described above, suggested the possibility of demonstrating antibody to the sera and danders of other animal species. Epithelial extracts and sera of several laboratory animals and domestic animals with which the patient had been in contact were linked to human R.B.C. in the same manner as mouse dander and mouse

serum. G.A.U. serum agglutinated most of the dander-linked cells except for dog and beef danders (Table III). Beef and dog danders had given minimal but positive direct skin tests.[1] The negative skin tests and hemagglutination tests must be interpreted with caution, however, since they may be caused by the weakness of the particular dander extract used.

G.A.U. serum also consistently agglutinated cells linked to all the mammalian sera tested except human sera (Table IV). Dog serum and beef serum,

TABLE IV. AGGLUTINATION BY G.A.U. SERUM OF R.B.C. ATTACHED TO ANIMAL SERA

	TITER OF G.A.U. SERUM	
ANIMAL SERA	B.D.B. METHOD	TANNED CELL HEMAGGLUTINATION METHOD
1. Mouse	320	700
2. Rat	640	*
3. Guinea pig	160	700
4. Rabbit	40	*
5. Cat	80	80
6. Dog	320	700
7. Horse	320	*
8. Beef	640	700
9. Sheep	640	*
10. Hog	640	*
11. Rhesus monkey	40	*
12. Lion	80	*
13. Tiger	160	*
14. Indian elephant	40	*
15. Coyote	160	*
16. Human	0	*
17. Frog	0	*
18. Chicken	0	*
19. Snake	0	*
20. Alligator	0	*

*Tests not done.

in contrast to their respective dander extracts, provided positive hemagglutination by G.A.U. serum. Although cat serum had given entirely negative skin tests, distinct hemagglutination was seen when it was used as the antigen in vitro. Where human serum-linked red blood cells were used, normal rabbit serum diluted 1:100 or normal chicken serum (1:100) with buffered saline instead of NHS 1/100 was the diluent. The negative results thus could not be attributed to the presence of free human serum acting as an inhibiting agent. As a positive control, antihuman serum rabbit serum was used and produced agglutination of the human serum-linked R.B.C. in high titer. As shown in Table IV, somewhat higher titers were usually obtained with the tanned cell method. However, since no agglutination was observed with the tanned cell method when animal danders were used as the coating antigen, the B.D.B. method was chiefly used in this study.

Also listed in Table IV are the results of reactions of G.A.U. serum with sera of certain wild beasts (namely, lion, tiger, Indian elephant, and coyote) with which the patient had no known previous contact. Hemagglutination was noted in all cases. Of the species listed, tiger and elephant gave only weakly

TABLE V. AGGLUTINATION BY G.A.U. SERUM OF R.B.C. AZO-LINKED TO KIDNEY EXTRACT AND TO SERA

SPECIES	TITER OF G.A.U. SERUM	
	KIDNEY EXTRACT	SERUM
1. Beef	640	320
2. Hog	320	80
3. Monkey	0	40
4. Human	0	0

TABLE VI. SUMMARY OF INHIBITION STUDIES

FREE ANTIGEN MIXED WITH G.A.U. SERUM*	R.B.C. AZO-LINKED TO ANIMAL SERA LISTED BELOW								
	MOUSE	RAT	GUINEA PIG	DOG	CAT	HORSE	RABBIT	HOG	BEEF
1. Mouse	6,400	3,200	0	0	0	0	0	0	0
2. Rat	0	6,400	0	0	0	0	0	0	0
3. Guinea pig	0	0	3,200	0	0	0	0	0	0
4. Dog	0	0	0	25,600	0	0	0	0	0
5. Cat	0	0	0	0	25,600	0	0	0	0
6. Horse	0	0	0	0	3,200	3,200	0	0	0
7. Rabbit	0	0	0	0	0	0	25,600	0	0
8. Hog	0	0	0	0	0	0	0	3,200	0
9. Beef	0	0	0	0	0	0	0	0	1,600
10. Human	0	0	0	0	0	0	0	0	0

Numbers represent reciprocal of the highest dilution of antigen inhibiting the agglutination of serum-linked R.B.C. by G.A.U. serum.

O = No inhibition.

*Allowed to stand at room temperature for one hour prior to addition of the red blood cells coated with the various animal sera (antigens).

positive reactions to intradermal skin tests on this patient, whereas coyote and lion sera produced much stronger reactions. Furthermore, reagins to lion and coyote, but not to elephant and tiger, could be demonstrated by passive transfer studies.[1]

These antigen-linked erythrocytes were not agglutinated by normal human serum or by the sera of six other allergic patients who also had a positive clinical history of sensitivity to animals and positive intradermal skin tests to animal epidermal extracts. Serum of one patient sensitive to rat dander* did agglutinate red blood cells linked to rat dander extract but gave entirely negative reactions with rat serum and mouse serum.

In addition to the mammalian sera tested, the sera of frogs, chickens, snakes, and alligators were also used as antigens to coat R.B.C. Negative results were obtained in each case (Table IV). In order to determine whether or not these nonmammalian sera were actually linked to the R.B.C., appropriate immune sera (antichicken-serum rabbit serum and antialligator-serum rabbit serum) were employed and did produce positive agglutination.

Table V summarizes the findings obtained with tissue extract from beef, hog, monkey, and human kidney and compares the titers with those obtained with the serum from the corresponding species. Hemagglutination in high titers was noted with beef and hog kidney extract as the antigen, higher titers being obtained with the tissue extract than with the homologous serum. No aggluination was observed when monkey kidney was tried, although monkey-serum-linked R.B.C. gave distinct agglutination with G.A.U. serum. Human serum and human kidney extract failed to react with G.A.U. serum. In contrast, human kidney extract and human serum, when attached to R.B.C., were agglutinated by antihuman-serum rabbit serum in titers of 40 and 320 respectively. In experiments with human serum and kidney extracts, normal rabbit serum (rather than normal human serum) was used as the diluent.

Inhibition Tests.—Two types of inhibition studies were carried out. In one type, serial dilutions of the antigen (0.1 ml.) were added to an equal volume of 1:5 diluted antiserum (G.A.U.). After one hour at room temperature, 0.1 ml. of antigen-linked R.B.C. was added to each tube of this mixture. The tests were all read as described previously.

Table VI summarizes the results of many experiments of this type. The figures in the table represent the greatest dilution of antigen (animal sera) which completely prevented the hemagglutination reaction of cells coated with homologous and heterologous sera. For example, when mouse serum (antigen) was previously mixed with G.A.U. serum (antibody) and then red blood cells coated with mouse serum (antigen) were added to this mixture, complete inhibition of agglutination was observed in dilutions of mouse serum up to 1:6,400. It required twice that amount of mouse serum (dilution 1:3,200) to inhibit the reaction of G.A.U. serum with red blood cells coated with rat serum. Even concentrated mouse serum, however, did not prevent the agglutination by

*Kindly furnished by Dr. Frank J. Gilday.

G.A.U. serum of cells coated with the various other animal sera. Hence, it can be seen from these results that the homologous serum can inhibit this type of reaction in high dilutions, whereas, heterologous animal sera usually produce little or no inhibition except in two situations. Mouse serum inhibited agglutination of rat-serum-coated cells to a degree, but rat serum did not affect the agglutination of mouse-serum-coated cells. Likewise, horse serum prevented the agglutination of cat-serum-coated cells, but the cat serum did not influence the agglutination of horse-serum-coated cells. These results with rat and mouse sera closely parallel the findings of reagin exhaustion studies described in our previous article.

In the second type of inhibition study, various dilutions of antigen as inhibiting agents were added to serial dilutions of antiserum and the mixtures were allowed to stand at room temperature for one hour. The antigen-linked red cells were then added to these mixtures, and the tests were read as before. The volume of each of the components was 0.1 ml.

The results of one quantitative inhibition test of this type are shown in Table VII. When mouse serum diluted 1:1,000 was added to G.A.U. serum

TABLE VII. QUANTITATIVE INHIBITION EXPERIMENT

DILUTIONS OF G.A.U. SERUM (ANTIBODY)	MOUSE SERUM DILUTIONS (INHIBITING ANTIGEN)*							
	0 (CONTROL)	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	UNDILUTED
1. 1:5	++++	++++	++++	++++	–	–	–	–
2. 1:10	++++	++++	+++	–	–	–	–	–
3. 1:20	++++	+++	++	–	–	–	–	–
4. 1:40	++	++	–	–	–	–	–	–
5. 1:80	++	–	–	–	–	–	–	–
6. 1:160	+	–	–	–	–	–	–	–
7. 1:320	–	–	–	–	–	–	–	–
8. Saline	–	–	–	–	–	–	–	–

*Added to various dilutions of G.A.U. serum (antibody) and allowed to stand at room temperature for one hour prior to addition of the R.B.C. azo-linked to mouse serum.

(antibody) 1:5 dilution prior to the addition of the mouse-serum-coated R.B.C., complete inhibition of agglutination was obtained. A dilution of 1:1,000,000 of mouse serum was sufficient to neutralize this reaction when G.A.U. serum was diluted 1:80. Similar tests with each of the various animal sera as inhibiting antigens and their respective serum-coated red cells, as well as heterologous inhibition tests, were likewise performed.

A comparison of the effect of inhibition of agglutination by homologous and heterologous sera is summarized in Table VIII. Mouse serum and dog serum are compared in their effect on inhibiting agglutination by G.A.U. serum of R.B.C. linked to the same and to the other sera. In both instances a 1:1,000 dilution of the inhibiting antigen is sufficient to inhibit the agglutination of R.B.C. linked to the homologous serum. However, a 1:10 diluted antigen is needed to inhibit agglutination of R.B.C. linked to the heterologous serum in each case.

In contrast to these results, coyote serum and mouse serum were compared and the findings are summarized in Table IX. Here the reaction of G.A.U. serum with coyote-serum-linked RBC is inhibited equally as effectively by mouse serum (the heterologous antigen) as by coyote serum (the homologous antigen). Coyote serum did not completely inhibit the agglutination of mouse-serum-linked RBC.

TABLE VIII. SUMMARY OF MUTUAL INHIBITION BY MOUSE SERUM AND DOG SERUM ANTIGENS

COATING ANTIGEN	INHIBITING ANTIGEN	DILUTIONS OF INHIBITING ANTIGENS*							
		0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	UNDILUTED
Mouse serum	Mouse serum	160†	40	20	5	0	0	0	0
	Dog serum	320	80	80	80	80	80	0	‡
Dog serum	Dog serum	160	20	20	5	0	0	‡	‡
	Mouse serum	640	320	160	40	20	20	0	0

*Reciprocal of highest dilution of G.A.U. serum which agglutinates coated R.B.C.
†Added to G.A.U. serum and allowed to stand at room temperature before addition of coated R.B.C.
‡Tests not done.

Inhibition experiments were designed to determine whether or not different antibodies to antigens were present in either serum or dander extracts. No inhibition of agglutination by R.B.C. linked to mouse dander or mouse serum was seen. Nor are mouse-dander-coated cells inhibited by mouse serum agglutinated by mouse dander.

TABLE IX. SUMMARY OF MUTUAL INHIBITION BY MOUSE SERUM AND COYOTE SERUM ANTIGENS

COATING ANTIGEN	INHIBITING ANTIGEN	DILUTIONS OF INHIBITING ANTIGENS*							
		0	10^{-6}	10^{-5}	10^{-4}	10^{-3}	10^{-2}	10^{-1}	UNDILUTED
Mouse serum	Mouse serum	160†	40	20	5	0	0	0	0
	Coyote serum	160	40	80	20	10	10	‡	‡
Coyote serum	Coyote serum	40	40	20	10	0	0	0	‡
	Mouse serum	40	40	40	20	0	0	0	0

*Reciprocal of highest dilution of G.A.U. serum which agglutinates coated R.B.C.
†Added to G.A.U. serum and allowed to stand at room temperature before addition of coated R.B.C.
‡Tests not done.

DISCUSSION

The demonstration in the serum of an allergic patient, of a substance capable of producing agglutination of antigen-linked erythrocytes is unusual. Studies with allergic sera to demonstrate antipollen antibodies by this method have been reported by Coombs,[5] Feinberg,[2] Sehon,[3] Stavitsky,[8] Kallos,[4] and Kraepelian,[9] and their co-workers, but these reports are not in agreement. Coombs and associates found that sera of certain pollen-sensitive patients who had had previous pollen hyposensitization therapy caused the agglutination of red blood cells coated by pollen but not by the sera of untreated pollen-sensitive patients. Feinberg and his co-workers were able to demonstrate essentially the same phenomenon. Stavitsky and associates, on the other hand, obtained uniformly negative results using the sera of both treated and untreated patients, while Sehon, Gordon, and Rose could obtain positive hemagglutination in high

titers by the sera of both treated and untreated ragweed sensitive patients. Kallos and Kallos also reported positive results in pollen-sensitive patients, but Kraepelian and colleagues could not verify these studies.

These differences in results could very well be due to slight deviations and variations in techniques. These are rather exacting types of experiments, and often they are not reproducible unless very carefully done. The interpretation of the readings of the agglutination of red blood cells can also account for their inconsistent results. Some workers feel that any type of clumping, pattern, or ring formation different from the normal serum control is a positive test. In our studies, very definite agglutination had to be present before a test was recorded as positive (see section on methods and materials).

In the G.A.U. serum, antibodies to many animal epithelia were clearly revealed in vitro when this hemagglutination method, was used. The sera of the same animal species provided even stronger reactions. Although at first it was felt that the reaction with serum was due to the presence of a common species-specific antigen found in both epithelial extracts and serum of the particular species, cross-absorption tests later suggested that antigens involved in the two types of serologic reactions with G.A.U. serum are distinct.

Studies by Longcope and associates[10] indicated differences in antigenic properties between horse serum and horse dander extract. Several other investigators[11, 12] have definitely shown that animals sensitized to horse dander could be shocked by horse serum. However, the reverse did not occur—that is, animals sensitized to horse serum could not be shocked by horse dander. Recent work by Silver and Bookman[13] suggests some immunologic and chemical similarity. This reflects, of course, a very important clinical point, namely, that patients developing serum sickness do not become "horse asthmatics," whereas, "horse asthmatics" can react very violently to horse serum.

Quantitative inhibition hemagglutination techniques are excellent tools for studying the relationship of dander to serum. Additional studies are now in progress using antidander and antiserum rabbit sera as the testing antibodies, rather than the unique serum of our patient.

The wide range of animal species to which reactions could be demonstrated with G.A.U. serum was startling. All mammalian species could be shown to react antigenically, with the exception of human serum. Attempts to find species giving negative tests led to the inclusion of sera from animals with which the patient had no known contact, such as lions, tigers, elephants, and coyotes. Only when avian and reptilian sera were tried did G.A.U. serum fail to agglutinate serum-linked erythrocytes. Because of its hemagglutinating ability, even in high dilutions, and its specificity evidenced by the failure to react to human or nonmammalian sera, we tentatively regard the hemagglutinating factor in this patient's serum as a true antibody.

Kidney extracts from various species were next used as the antigens to determine whether or not the antibody was directed against tissue factors other than those present in serum and in skin. Again agglutination occurred except where human and monkey kidney extracts were used. The findings suggest that agglutination occurred because the tissue extracts contain the

antigen involved, rather than because of the presence of serum in the organ extract. Kidney extract gave somewhat higher titers in simultaneous tests than did homologous serum used as antigen.

Inhibition studies were performed to determine the degree of specificity of these reactions. Marked inhibition always occurred with the homologous free antigen when it was incubated with G.A.U. serum prior to the addition of antigen-linked R.B.C. In contrast, sera of other species were generally considerably less effective in neutralizing the reaction, indicating that predominantly species-specific antibodies determine each reaction. On the other hand, the fact that addition of heterologous sera was able to bring about a measureable decrease in the reactions of G.A.U. serum with sera of various mammals suggests the existence of somewhat similar antigenic groupings in each of these mammalian sera.

In a few instances (in particular, in the case of such unfamiliar species as the coyote) the reaction of G.A.U. serum can be neutralized by heterologous mouse serum as effectively as by the homologous coyote serum. This finding indicates the absence of species-specific antibodies against coyote serum. Hemagglutination of coyote-linked R.B.C. is presumably due solely to a stereochemical resemblance between coyote and other mammalian sera. Because of the lack of direct exposure, the patient presumably had no opportunity to produce species-specific coyote antibodies.

The validity of postulating separate antibodies for the various species studied is partly supported by the results of skin tests. A site passively sensitized with G.A.U. serum in a nonallergic recipient and exhausted by repeated testing with one antigen would react positively when a second antigen was used to retest this site.[1] Nevertheless, the experimental evidence available thus far suggests that the antibody detectable in hemagglutination tests is different from that responsible for the skin test reactions. The evidence for the differences is as follows: (1) the hemagglutination antibody is stable after prolonged heating at 56° C.; (2) hemagglutination reactions occur only with mammalian antigens, whereas the patient was found to give positive skin test reactions to snake, frog, and alligator serum.

Further studies are contemplated to determine the factor in G.A.U. serum which causes this broad spectrum of agglutination of the various mammalian sera. The possibility that these effects may have been nonspecific was considered. However, the negative results with human serum and nonmammalian sera point to the specificity of the reaction. Nonspecific clumping of R.B.C. may be observed in sera of patients who had high globulin levels (as in multiple myeloma). In order to rule out this possibility, protein determinations (albumin and globulin) and electrophoretic patterns of the patient's serum were studied. The results of these determinations were all within normal limits. A study of the sera of several members of this patient's family, some of whom are allergic, would also be valuable in an attempt to determine some genetic factor.

SUMMARY AND CONCLUSIONS

1. The serum of a patient clinically sensitive to several animal danders was studied immunologically.

2. A substance was present in this patient's serum which caused agglutination in vitro of red blood cells coated with all mammalian sera tested except human serum. Similar reactions were noted with various animal danders and tissue extracts coated on red blood cells.

3. This substance is thermostable and thus differs from the patient's serum reagin (Prausnitz-Küstner) antibody.

4. Each reaction seemed predominantly species specific, with the exception of certain species with which the patient had no direct contact. In these instances, the reaction appeared to be caused entirely by antigenic cross-reaction with other mammalian sera.

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REFERENCES

1. Arbesman, C. E., Beede, R. B., and Rose, N. R.: Sensitivity to Animals: A Case Report With Immunologic Studies. I. Clinical Aspects, J. ALLERGY **29**: 130, 1958.
2. Feinberg, R. J., Davison, J. D., and Flick, J. A.: The Detection of Antibodies in Hay Fever Sera by Means of Hemagglutination, J. Immunol. **77**: 279, 1956.
3. Sehon, A. H., Gordon, J., and Rose, B.: Antibodylike Factor in Serums of Ragweed-Sensitive Individuals Shown in Vitro, Science **125**: 3248, 1957.
4. Kallos, P., and Kallos, Deffner: Specific Serum-Agglutination of Corpuscles (Collodium-Pellets on Erythrocytes Coated With Allergenic Material), Preliminary Report, Internat. Arch. Allergy and Applied Immunology, vol. 2, fasc. 1, p. 70, 1956.
5. Coombs, R. R. A., Howard, A. N., and Mynors, L. S.: A Serological Procedure Theoretically Capable of Detecting Incomplete or Non-Precipitating Antibodies to Soluble Protein Antigens, Brit. J. Exper. Path. **34**: 525, 1953.
6. Pressman, D., Campbell, D. H., and Pauling, L.: The Agglutination of Intact Azo-Erythrocytes by Antisera Homologous to the Attached Groups, J. Immunol. **44**: 101, 1942.
7. Boyden, S. V.: The Adsorption of Protein on Erythrocytes Treated With Tannic Acid and Subsequent Hemagglutination by Anti Protein Sera, J. Exper. Med. **93**: 107, 1951.
8. Stavitsky, A. B., and Arquilla, E. R.: Micro Methods for the Study of Protein and Antibodies. III. Procedure and Application of Hemagglutination and Hemagglutination Inhibition Reactions With Bis-Diazotized Benzidine and Protein Conjugated Red Blood Cells, J. Immunol. **74**: 306-312, 1955.
9. Kraepelian, S., Sievert, B., and Wettergren, S.: Experiments With a New Agglutination Method in Ascertaining Antibodies in Allergic Subjects, Acta paediat. **42**: 412, 1953.
10. Longcope, W. T., O'Brien, D. P., and Perlzweig, W. A.: The Antigenic Properties of Extracts of Horse Dander. I. Active Sensitization of Guinea Pigs to Horse Dander Extract, J. Immunol. **10**: 599, 1925.
11. Tuft, L.: Further Studies in Serum Allergy. VI. Antigenic Relationship Between Horse Dander and Horse Serum Sensitivity, J. ALLERGY **6**: 25, 1934.
12. Forestor, G. F.: Is There an Immunologic Relationship Between Horse Serum and Horse Dander? J. Exper. Med. **47**: 903, 1928.
13. Silver, D. Z., and Bookman, R.: Studies on the Nature of Allergenic Material in Animal Danders and Pollens, J. ALLERGY **27**: 127, 1956.

Addendum

After this manuscript was prepared, a sample of serum from a patient sensitive to mouse dander was received from Drs. Sorrell and Gottesman (*Ann. Allergy,* November, 1957). This serum also reacted with mouse serum and several other mammalian sera.

AN APPRAISAL OF ORAL AND AEROSOLIZED METHYLPHENIDATE HYDROCHLORIDE (RITALIN) IN OBSTRUCTIVE PULMONARY DISEASE

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REPORTS that methylphenidate hydrochloride (Ritalin*) is an effective psychoanaleptic agent have been amply confirmed.[1-3] A group of patients with various allergic conditions had been on daily oral doses of Ritalin for weeks to months in an earlier study in order that we might follow the effects of the drug on the formed elements of blood and on renal function. No significant abnormalities were observed.[4] A by-product, however, of these observations was the clinical impression that Ritalin appeared to have possible bronchodilator activity.

The present report describes our experience with Ritalin in patients with bronchial asthma and pulmonary emphysema.

RITALIN BY MOUTH

Methods.—In the period between April, 1956, and March, 1957, twenty patients with bronchial asthma served as subjects for this study. There were fourteen male patients and six female patients, ranging in age from 14 to 65 years with an average age of 45 years. All had the perennial variety of asthma, which was highly reversible, and all were having mild to moderate symptoms at the time treatment with one or the other preparation was started. Three patients were taking either cortisone or another steroid analogue during the period of study, and twelve patients were receiving treatment with allergenic extracts. The test subjects were instructed to avoid the use of other medications for relief of their asthma.

The following drugs were evaluated: (1) Ritalin, 5 mg.; (2) ephedrine sulfate, 25 mg.; and (3) lactose (placebo). The three preparations were coded by color, so that they were readily distinguishable from one another, and they were dispensed in a single bottle. Every patient in this study was shown to be able to distinquish the three colors with ease. Neither the patient nor the doctor was aware of the identity of any of the preparations. In order to obtain the cooperation of the test subjects, it was necessary to identify the tablets as medication intended for the relief of their asthma. The following instructions were given all patients: "When symptoms occur, take any one of the colored tablets; if relief follows, continue taking the tablets of the color selected every four hours

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as required until none remain. On the other hand, if relief is not satisfactory, change to a tablet of another color. Continue in this manner until all three preparations have been tried."

Subjects were usually seen at seven- to fourteen-day intervals over a period of weeks. Based on the patients' statements, the following criteria were used in evaluating the results: *marked relief,* if complete or almost complete relief of symptoms occurred for four or more hours; *moderate relief,* if relief was 50 to 75 per cent and lasted for two or more hours; *slight relief,* if relief was between 25 and 50 per cent and lasted one to two hours; and *no relief,* if relief was less than 25 per cent or if the duration of effectiveness was short.

When the investigation was completed and the data were tabulated, the code was "broken" and the medications were found to be as follows:

Red = Ritalin
Green = Ephedrine sulfate
Yellow = Lactose (placebo)

Inspection of the data (Table I) clearly shows that it was only among those asthmatic patients receiving ephedrine that a majority derived marked to moderate relief; conversely, a high proportion of those receiving Ritalin derived no benefit. The placebo occupied a somewhat intermediate place so far as effectiveness was concerned. Furthermore, when the response scale was arbitrarily assigned the values of 3 (marked relief), 2 (moderate relief), 1 (slight relief), and 0 (no relief) and a mean index of "perceived effectiveness" was computed for each drug, the mean values were found to be as follows: Ritalin, 0.45; ephedrine, 1.80; and the placebo, 0.90. Ephedrine had the highest index of "perceived effectiveness," its over-all average index being close to the moderate improvement "scale point." The differences among the preparations appear to be highly significant. Color preference and the order of administration of the drugs, however, did not appear to be significant factors. (See Table I.)

TABLE I. EFFECTIVENESS OF THE COLOR-CODED PREPARATIONS AND ORDER OF CHOICE IN TWENTY PATIENTS WITH BRONCHIAL ASTHMA

DRUG AND COLOR	DEGREE OF RELIEF				CHOICE		
	MARKED	MODERATE	SLIGHT	NONE	FIRST	SECOND	THIRD
Ritalin (Red)	0	2	5	13	5	12	3
Ephedrine (Green)	7	6	3	4	7	3	10
Placebo (Yellow)	2	3	6	9	8	5	7

RITALIN AS AN AEROSOL

Twenty patients with bronchial asthma, six of whom had an irreversible component to their obstructive pulmonary disease (emphysema), were exposed to aerosolized Ritalin, 5 mg. per milliliter. There were six female patients and fourteen male patients, with ages ranging from 8 to 78 years. The average age

was 47.6 years, and the median was 53 years. With one exception, the inhalation of Ritalin was followed by isopropylarterenol (Isuprel), 5 mg. per milliliter, given in the same manner. The effectiveness of Isuprel as a bronchodilator agent is well established and is used in this study as the standard reference drug.

The tests were carried out with patients seated and at rest, following which a number of preliminary measurements were made to establish a base line. Vital capacities and maximal expiratory flow rates were obtained simultaneously and graphically recorded. The figures given in Table II represent the largest value observed after two or three successive efforts showed no further increase. The method has been described in detail elsewhere.[5-7]

A No. 40 DeVilbiss nebulizer was used, with air delivered at the nebulizer by a compressor controlled by occlusion of an open Y tube. Exposure to the drug was carried out by having the patient inhale deeply with the nebulizer held before his open mouth, during which time the Y tube was closed. With few exceptions, each subject was given ten inhalations of the drug under appraisal. When more than one drug was studied, the patient was given a rest period of at least ten minutes between exposures.

The nature of the aerosolized medications was unknown to the patients. However, the patients probably believed that they were receiving a form of therapy.

Results.—As shown in Table II, following Ritalin there was an increase of 200 c.c. or more in the vital capacity in only four subjects (Cases 4, 8, 13, and 19). Either no significant increase occurred or there was a frank decrease in the vital capacity in the remaining sixteen patients. In Cases 9, 10, 11, and 13 the volume expired in the first second increased by 200 c.c. or more, and in the remaining sixteen patients either the increase was less than 200 c.c. or there was an actual decrease in this volume. The average increase in the vital capacity for all the subjects was only 80.5 c.c., with a decrease of 22 c.c. in the volume expired in the first second.

In nineteen of the twenty patients who received Isuprel by aerosol following Ritalin, ten showed an increase of 200 c.c. or more in the vital capacity, based on the values obtained following Ritalin. In six patients the increase was less than 200 c.c., in one there was no change, and in two there was a decrease. Corresponding figures for the volume expired in the first second were as follows: thirteen patients had an increase of 200 c.c. or more; two had an increase of less than 200 c.c.; two had no change; and in two there was a decrease. The average changes in vital capacity and in the volume expired in the first second for the group were 268 c.c. and 292 c.c., respectively. Thus, when given in this manner, Isuprel was far superior to Ritalin in its bronchodilator activity.

RITALIN GIVEN AFTER THE ADMINISTRATION OF ISUPREL

Seventeen patients with bronchial asthma, seven of whom had an irreversible component to their obstructive pulmonary disease (emphysema), were exposed to aerosolized Ritalin after they had been given Isuprel. In one patient,

TABLE II. RITALIN GIVEN BY AEROSOL BEFORE ISUPREL

PATIENT	AGE	SEX	DIAGNOSIS	CONTROL VALUES VITAL CAPACITY		CHANGE FOLLOWING RITALIN VITAL CAPACITY		CHANGE FOLLOWING ISUPREL* VITAL CAPACITY	
				TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)
1. E. M.	43	Female	Asthma	2,000	1,500	+100	—	+400	+450
2. S. F.	62	Male	Asthma and emphysema	4,200	2,200	- 50	-200	+150	+500
3. P. T.	17	Male	Asthma	4,250	4,000	+ 50	-100	-250	-100
4. J. R.	58	Male	Asthma and emphysema	2,050	1,200	+550	+150	+100	-250
5. G. J.	70	Male	Asthma	2,450	1,300	-200	-150	+250	+100
6. A. P.	35	Female	Asthma	2,650	2,000	+150	+100	-200	+150
7. D. H.	16	Male	Asthma	3,900	3,300	+100			
8. J. C.	17	Male	Asthma	4,350	3,500	+350	-200	+150	+800
9. J. K.	34	Male	Asthma	3,300	2,200	-200	+300	+600	+600
10. J. C.	60	Male	Asthma and emphysema	2,600	1,100	+100	+200	+1,000	+200

11. I. S.	53	Male	Asthma	2,500	1,300	+100	+250	+900	+750
12. C. R.	78	Male	Asthma	1,600	1,400	—	-600	+100	+600
13. A. R.	38	Male	Asthma	4,200	2,900	+200	+200	+100	+200
14. I. L.	54	Female	Asthma	2,050	1,450	-150	-250	+400	+200
15. M. G.	72	Male	Asthma and emphysema	2,400	1,500	-100	-100	+100	—
16. A. U.	50	Female	Asthma	2,150	1,400	+150	+100	+500	+500
17. J. C.	8	Male	Asthma	1,100	1,000	+100	—	+300	—
18. A. C.	54	Female	Asthma	2,400	1,700	-300	-200	+200	+200
19. M. C.	72	Male	Asthma and emphysema	1,500	1,000	+500	+150	—	+350
20. A. S.	63	Female	Asthma and emphysema	1,800	1,200	—	—	+300	+300
			Average values	2,672	1,857	+80.5	- 22	+268	+292

*Over and above that produced by Ritalin.

TABLE III. RITALIN GIVEN BY AEROSOL AFTER ISUPREL

PATIENT	AGE	SEX	DIAGNOSIS	CONTROL VALUES VITAL CAPACITY		CHANGE FOLLOWING			
						ISUPREL VITAL CAPACITY		RITALIN* VITAL CAPACITY	
				TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)
1. J. C.	9	Male	Asthma	1,700	1,300	—	-100	+200	+300
2. T. S.	13	Male	Asthma	2,600	2,100	+100	—	—	—
3. R. C.	13	Male	Asthma	2,200	1,450	-200	+350	+200	+200
4. I. L.	54	Female	Asthma	2,500	2,000	+200	-200	+100	+200
5. J. R.	58	Male	Asthma and emphysema	2,100	1,200	+200	-200	+400	—
6. M. G.	72	Male	Asthma and emphysema	2,100	1,300	+300	+100	+200	—
7. H. H.	52	Male	Asthma	2,900	1,900	+600	+200	—	+100
8. S. F.	62	Male	Asthma and emphysema	3,900	1,800	+800	+600	+300	+100
9. S. F.	62	Male	Asthma and emphysema	3,200	1,400	+1,100	+900	+600	+200

10. E. K.	70	Female	Asthma	2,800	1,800	+300	+700	—	—
11. M. T.	43	Male	Asthma	3,300	1,600	+900	+1,000	-200	—
12. S. K.	59	Male	Asthma and emphysema	1,650	1,000	+350	+100	- 50	+ 50
13. A. S.	62	Male	Asthma and emphysema	1,800	1,400	+300	+100	+300	+100
14. M. C.	72	Male	Asthma and emphysema	1,900	1,300	+300	+200	-100	+100
15. J. S.	11	Male	Asthma	2,700	2,350	+100	+150	-100	—
16. A. C.	54	Female	Asthma	2,200	1,900	+400	+100	—	—
17. G. C.	50	Female	Asthma and emphysema	1,260	900	+570	+175	-230	+ 25
18. A. F.	34	Male	Asthma	4,350	3,100	+550	+600	+200	+200
			Average values	2,508	1,655	+382	+266	+101	+ 88

*Over and above that produced by Isuprel.

measurements were carried out on two different occasions (experiments No. 8 and No. 9 in Table III). There were twelve male patients and five female patients in the group, with ages ranging from 9 to 72 years. The average age was 46 years.

When Isuprel was given first (Table III), the vital capacity increased by 200 c.c. or more in fourteen of the eighteen experiments; the volume expired in the first second also increased by 200 c.c. or more in eight of the studies. In the remaining experiments, with one exception, there was either an increase of less than 200 c.c., or there was an actual decrease in these volumes. Upon Ritalin administration, the vital capacity showed a further improvement of 200 c.c. or more, based on the values obtained with Isuprel in eight of the experiments; one increased by 100 c.c.; in four there was no change; and in the remaining five there was a decrease. Along with this, the volume expired in the first second increased by 200 c.c. or more in five of the eighteen experiments, in six experiments the increase was less than 200 c.c., and in the remaining seven measurements there was no change.

For the entire group, when Isuprel was given first, the average increase in vital capacity was 382 c.c., with an average increase of 266 c.c. expired in the first second. When Ritalin followed Isuprel, the average changes in vital capacity and in the volume expired in the first second over and above that produced by Isuprel were 101 c.c. and 88 c.c., respectively.

Since the increase in vital capacity and rate of expiration observed when Ritalin followed Isuprel might be attributable to a continuing effect of Isuprel, saline was substituted for Ritalin in ten experiments. In these studies, saline following Isuprel produced, on the average, a change in vital capacity of +3 c.c. and in the rate of expiration of +67 c.c.

DISCUSSION

That clinical impressions should be accepted with the proverbial "grain of salt" is underscored by the results of this study. In effect, an opinion based on uncontrolled evaluation of subjective responses, which led to this study, was not borne out by objective tests of pulmonary function. Patients apparently interpreted the mood-elevating property of Ritalin as effectively relieving the symptoms of obstructive pulmonary disease. When Ritalin was evaluated by a double "unknowns" technique and the findings were analyzed statistically, no evidence could be adduced to indicate an effect of Ritalin in reducing the manifestations of bronchial asthma. This is evident in the response scale. Thirteen out of twenty patients rated ephedrine as giving marked to moderate relief and only two out of twenty patients taking Ritalin described moderate relief. The small number of patients who reported relief from the placebo was not unexpected.[8]

Ritalin given as an aerosol produced, on the average, no increase in the rate of expiration and only an equivocal increase in vital capacity when given alone. Following Isuprel, however, and therefore at a time when in almost every instance a considerable bronchodilator effect had already been produced, Ritalin induced again, on the average, a slight increase in the speed of expiration and

vital capacity. In view of the results when saline was substituted for Ritalin, however, the changes in the speed of expiration may be attributable to a delayed effect of Isuprel. Since the delayed effect of Isuprel on the vital capacity was negligible, the increase in this volume, following Ritalin, may have some significance.

SUMMARY AND CONCLUSIONS

1. Twenty patients with bronchial asthma compared the effectiveness of three oral preparations. These were methylphenidate hydrochloride (Ritalin), 5 mg.; ephedrine sulfate, 25 mg.; and a placebo (lactose). Compared to ephedrine, Ritalin was found to be an ineffective agent in the management of bronchial asthma.

2. When the two drugs, Ritalin and isopropylarterenol (Isuprel), were given in sequence by inhalation in a concentration of 0.5 per cent, no significant changes in vital capacity and expiratory flow rate were observed when Ritalin was given first. However, when Ritalin followed Isuprel, an increase in the vital capacity and the volume expired in the first second occurred over and above that produced by Isuprel, but these changes were of doubtful significance.

3. Untoward side effects from aerosolized or oral Ritalin were not observed.

REFERENCES

1. Ferguson, J. T.: Treatment of Reserpine-Induced Depression With New Analeptic, Phenidylate, Ann. New York Acad. Sc. **61:** 101, 1955.
2. Carter, C. H.: Effects of Reserpine and Methyl-Phenidylacetate (Ritalin) in Mental Defectives, Spastics, and Epileptics, Am. Psychiatric Association, Psychiatric Research Reports, No. 4, p. 44, 1956.
3. Ferguson, J. T., Linn, W. V. Z., Sheets, J. A., Jr., and Nickels, M. M.: Methylphenidate (Ritalin) Hydrochloride Parenteral Solution, J. A. M. A. **162:** 1303, 1956.
4. Schiller, I. W.: Unpublished observations.
5. Schiller, I. W., and Lowell, F. C.: The Effect of Drugs in Modifying the Response of Asthmatic Subjects to Inhalation of Pollen Extracts as Determined by Vital Capacity Measurements, Ann. Allergy **5:** 564, 1947.
6. Lowell, F. C., and Schiller, I. W.: Measurement of Changes in Vital Capacity as a Means of Detecting Pulmonary Reactions to Inhaled Aerosolized Allergenic Extracts in Asthmatic Subjects, J. ALLERGY **19:** 100, 1948.
7. Franklin, W., Michelson, A. L., Lowell, F. C., and Schiller, I. W.: Clinical Value of a Tracing of Forced Expiration (Expirogram), New England J. Med. **253:** 799, 1955.
8. Schiller, I. W., and Lowell, F. C.: A Comparative Study of Sc-4959 (1-n.-propyl-3-ethyl-5-chloro-6-aminotetrahydropyrimidinedione) and Hyadrine in Bronchial Asthma, J. ALLERGY **27:** 68, 1956.

METHYLPHENIDATE HYDROCHLORIDE (RITALIN) IN OBSTRUCTIVE PULMONARY DISEASE

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PRELIMINARY pharmacologic data[1] and clinical experience[2] with methylphenidate hydrochloride (Ritalin*), a new analeptic compound, indicated possible bronchodilator activity and prompted the evaluation of this drug in patients with obstructive pulmonary disease.

METHODS AND MATERIALS

Eleven experiments were performed in seven patients with bronchial asthma. Four of the patients were women and three were men; their ages ranged from 19 to 60 years. All had had mild to moderately severe symptoms

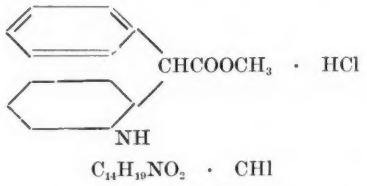

Fig. 1.—Ritalin (methyl-a-phenyl-2-piperidineacetate hydrochloride).

for five years or more. Lung volumes and ventilatory measurements were made with the patient seated and at rest, breathing through a rubber mouthpiece into a closed spirometer system recording on two kymographs.[3-5] The first kymograph was run at a speed of 5 cm. per minute to obtain the conventional spirogram. The second was run intermittently at 60 cm. per minute to obtain a measure of the maximal expiratory flow rate. Oxygen was supplied to the closed system to replace that consumed by the subject, and carbon dioxide was absorbed by a soda-lime scrubber.

Determinations of the minute volume, the vital capacity, and the volume expired in the first second during the performance of the vital capacity were made before, immediately after, and at ten- to fifteen-minute intervals following the rapid intravenous administration of 2.5 to 10 mg. of Ritalin. During the experiments the blood pressure and pulse were recorded and compared to

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their base line measurements. The nature of the intravenous medication was unknown to the patients, but most of them believed that they were receiving a form of therapy.

RESULTS

The results are shown in Table I. With the exception of two of the eleven experiments (Nos. 3 and 10), a significant increase in both the vital capacity and the expiratory flow rate was seen following administration of Ritalin. In three experiments the maximal increase in the vital capacity and volume expired in the first second occurred soon after the administration of the drug. In another experiment (No. 9) the vital capacity reached its peak value in a few minutes, but the maximal volume expired in the first second was not reached for ten minutes or more, at a time when the vital capacity again approximated its base line measurement. In the remaining experiments the greatest values for these volumes were not seen for ten minutes or more.

With the exception of experiment 1, the minute volume increased following administration of Ritalin; this increase is best illustrated in experiments 3, 6, 7, and 11. Changes in both rate and tidal volume accounted for the increase in the minute ventilation. The rise in ventilation occurred within a few minutes after administration of the drug, and it generally lasted throughout the experiment.

Observations on the cardiovascular effects were possible in nine of the eleven experiments. The effect on the blood pressure was variable. In four instances (experiments 2, 3, 7, and 11) a transient rise in blood pressure, with an increase in both the systolic and diastolic pressure, was noted; in the remaining experiments no significant alteration was observed.

With only one exception (experiment 11), which will be discussed later, there were no important alterations in the pulse rate in the remaining studies.

DISCUSSION

Intravenously administered Ritalin appears to have bronchodilator activity. Although immediately following the injection of Ritalin the vital capacity and expiratory flow rate increased by an average of only 200 c.c. and 130 c.c., respectively, these values showed a further increase to 545 c.c. and 405 c.c. from the base line values within ten to fifteen minutes. These findings are in contrast to another report[2] in which no evidence could be adduced to indicate an effect of orally administered Ritalin in reducing the manifestations of bronchial asthma. In the same investigation, the effectiveness of aerosolized Ritalin as a bronchodilator agent was only equivocal.

The minute volume increase from 20 to 300 per cent in all but one of the seven subjects, indicating that Ritalin has a stimulating effect on the central nervous system. Despite this, the patients felt that they could breathe more deeply and with less effort after the intravenous medication. This may be regarded as added evidence of bronchodilatation, in view of the simultaneous occurrence of increased ventilation and subjective relief of bronchial obstruction.

TABLE I. PULMONARY AND CARDIOVASCULAR EFFECTS OF RITALIN IN PATIENTS WITH BRONCHIAL ASTHMA

EXPERIMENT NO., PATIENT, AGE, AND SEX	HEIGHT (CM.)	DATE	INTRAVENOUS RITALIN																COMMENTS
			BASE LINE MEASUREMENTS					IMMEDIATE EFFECTS						10-15 MINUTES AFTER ADMINISTRATION					
					VITAL CAPACITY						VITAL CAPACITY					VITAL CAPACITY			
			BLOOD PRESSURE	PULSE	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	MINUTE VOLUME (L./MIN.)	DOSE (MG.)	BLOOD PRESSURE	PULSE	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	MINUTE VOLUME (L./MIN.)	BLOOD PRESSURE	PULSE	TOTAL (C.C.)	VOLUME FIRST SECOND (C.C.)	MINUTE VOLUME (L./MIN.)	
1. E. H., 19, female	153	4/17/56			1,000	450	9.1	7.5			1,400	600	8.1						Patient felt hot, had dry throat and stuffy nose; she became nauseated and vomited
2. E. W., 28, female	153	5/17/56	120/80	128	700	500	18.9	10	130/90	120	1,400	700	18	140/80	120	1,550	750	24	Patient had an ephedrine tablet 90 minutes before experiment; less tightness of chest
3. "		5/31/56	110/60	120	2,200	1,100	14.4	10	136/90	120	2,200	1,100	25.3	130/84	120	2,300	1,200	22	Patient able to breathe easier and more rapidly
4. M. W., 39, female	165	4/17/56			2,400	1,900	6.1	2.5			2,650	2,100	6.6						
5. "		4/25/56	110/84	80	2,400	1,800	5.6	5			2,700	2,000	7.6	118/80	84	2,550	2,000	6	
6. R. G., 59, male	178	5/22/56	160/100	76	2,750	2,050	6	5	154/100	76	2,700	2,100	14	150/100	80	3,050	2,250	20	Patient breathed easier, although he had slight palpitation

7. S. F., 60, male	175	5/29/56	140/90	76	3,300	1,800	14	10	170/110	76	3,500	2,200	28	156/100		4,100	2,200	21	Disappearance of "air trapping"
8. E. H., 22, female	165	7/3/56	90/64	96	4,200	2,800	7.2	5	92/64	86	4,300	2,800	7.2	90/68	96	4,800	3,300	10.8	Patient had an ephedrine tablet three hours before experiment
9. "		9/13/56	110/80	80	1,850	750	13.5	5	116/82	80	2,450	900	14	116/82	88	1,840	1,200		Relief of dyspnea
10. H. A., 45, male	171	7/10/56	120/82	80	3,300	2,300	8	5	118/84	80	3,000	2,300	12	112/86	88	3,400	2,400	14	Deep inspiration facilitated
11. "		8/16/56	130/90	72	4,000	2,700	7.7	10	164/100	100	4,000	2,800	19.8	150/90	106	4,300	3,200	9	Became jittery within 6 minutes following administration of drug

In the experimental animal, Ritalin blocks the pressor response to ephedrine and amphetamine but potentiates the pressor response of epinephrine and norepinephrine.[1] In the studies reported here, Ritalin produced a modest and transient rise in blood pressure in four of the eleven experiments.

The subject (experiment 11) in whom a significant rise in pulse rate occurred received 10 mg. of Ritalin. Immediately after the drug was injected, his pulse rose from a base line value of 72 per minute to 100 per minute, and six minutes later he began to complain of feeling "very jittery"; in ten minutes his pulse was 106 per minute. Reassurance and oral administration of a barbituate lessened the nervous reaction.

Following the administration of 7.5 mg. Ritalin intravenously, one subject (experiment 1) complained of a stuffy nose and dry throat. She became nauseated and vomited but recovered fully in thirty minutes. At this time, we are in doubt as to the part played by Ritalin in this reaction.

SUMMARY

Eleven experiments were carried out in seven patients with bronchial asthma. Methylphenidate (Ritalin) was given intravenously in a dosage of 2.5 to 10 mg., and the lung volumes, ventilation, and certain cardiovascular effects were measured before and after the administration of the drug. In general, Ritalin increased the vital capacity and the maximal expiratory flow rate and actively stimulated both the depth and the rate of respiration. The effect of Ritalin on the cardiovascular apparatus was inconstant and, for the most part, moderate and transient.

REFERENCES

1. Maxwell, R. A., Plummer, A. J., Ross, S. D., Pavtas, J. J., and Dennis, A. D.: Antihypertensive Effects of the Central Nervous Stimulant, Methylphenidate, Arch. Int. Pharmacodyn. **CXII**: Nos. 1 and 2, 1957.
2. Schiller, I. W., and Lowell, F. C.: An Appraisal of Oral and Aerosolized Methylphenidate (Ritalin) Hydrochloride in Obstructive Pulmonary Disease, J. ALLERGY **29**: 151. 1958.
3. Lowell, F. C., Schiller, I. W., and Lowell, A.: The Use of a Closed System in the Study of Asthma and Emphysema, J. ALLERGY **23**: 335, 1952.
4. Schiller, I. W., and Lowell, F. C.: Pulmonary Function in Bronchial Asthma, J. ALLERGY **25**: 364, 1954.
5. Michelson, A. L., and Schiller, I. W.: The Effect of ω-Methoxy-poly(ethyleneoxy) ethyl-p-Butylaminobenzoate (Tessalon) on Ventilation and Lung Volumes, J. ALLERGY **28**: 514, 1957.
6. Ferguson, J. T., Finn, F. V. Z., Sheets, J. A., Jr., and Nickels, M. M.: Methylphenidate Ritalin) Hydrochloride Parenteral Solution, J. A. M. A. **162**: 1303, 1956.

THE TRACHEAL STRIP: OBSERVATIONS ON THE RESPONSE OF TRACHEAL MUSCLE

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A METHOD of study of the pharmacologic effect of drugs on guinea pig tracheal muscle was introduced in 1947 by Castello and de Beers.[1] This preparation has been called the "tracheal chain" and has proved valuable for demonstrating the effects of bronchoconstricting and bronchodilating drugs and antagonists of these drugs. The tracheal chain preparation consists of ten to twelve tracheal rings tied together, suspended in suitable physiologic solution, and tied to a writing lever in a manner suitable for recording the tracheal muscle contractions on a moving kymograph. The object of the present article is to describe a modification of this preparation and present the results of some studies on guinea pig tracheal muscle conducted with the modified preparation. These studies include several different attempts to sensitize passively the guinea pig trachea and the determination of the effect of hydrocortisone on smooth muscle constriction.

METHOD

The tracheal strips were prepared from the tracheas of normal female guinea pigs. The guinea pigs were killed by a blow on the head, and the tracheas were removed and carefully cleaned of connective tissue. A wooden applicator stick was inserted into the trachea and fixed with a pin to one end. The trachea was next cut with a sharp scalpel blade in a spiral fashion to produce a thin strip of tracheal tissue (Fig. 1). The strip of tracheal tissue was then placed in a bath of Ringer-Locke solution through which 95 per cent oxygen and 5 per cent carbon dioxide was continually bubbled. The temperature was maintained at 37° C. by an electric hot plate beneath the bath containing the tracheal strip. In those experiments in which a control from the same animal was necessary, the tracheal strip was divided into two or three equal lengths prior to suspension in the Ringer-Locke solution. Contractions of the tracheal strip were recorded on a smoked kymograph drum moving at 4 cm. per minute.

RESULTS

Contraction of Sensitized Trachea With Egg Albumin.—A guinea pig was sensitized with 4 mg. of crystallized egg albumin, injected intraperitoneally on three occasions at three-day intervals. The tracheal strip from this guinea pig contracted when 4 mg. of egg albumin was added to the bath (Fig. 2).

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The Antispasmodic Effect of Epinephrine and Benadryl.—The addition of Benadryl, 3 mg. per 100 c.c., or epinephrine, 0.3 mg. per 100 c.c., completely antagonized the contraction of a tracheal strip from egg albumin as described above or from histamine, 0.1 mg. per 100 c.c.

Contraction From Histamine Following Maximal Contraction With Antigen.—Following maximal contraction of a trachea sensitized to egg albumin as described above, the addition of histamine, 0.1 mg. per 100 c.c., resulted in further contraction of the tracheal strip (Fig. 2).

Fig. 1.—A guinea pig trachea is on the applicator stick and partially sectioned into the tracheal strip.

Failure of Hydrocortisone Hemisuccinate to Modify or Prevent the Contraction of the Guinea Pig Trachea.—The trachea of a guinea pig sensitized to egg albumin was sectioned into two equal segments. One of the tracheal strips was suspended in the bath, and egg albumin was added with resultant contraction of the trachea. The addition of hydrocortisone hemisuccinate, 5 mg. per 100 c.c., did not modify this response. The second portion of the trachea was incubated with hydrocortisone, 5 mg. per 100 c.c., for forty-five minutes. It was then suspended in the bath and egg albumin was added. A contraction similar to that noted with the untreated portion of trachea occurred (Fig. 3). This experiment was repeated, using hydrocortisone concentrations of 10 mg. per 100 c.c. and 30 mg. per 100 c.c. without modification of the tracheal response to the antigen. The same pretreatment with hydrocortisone failed to modify contractions caused by histamine, 0.1 mg. per 100 c.c., in the normal guinea pig trachea.

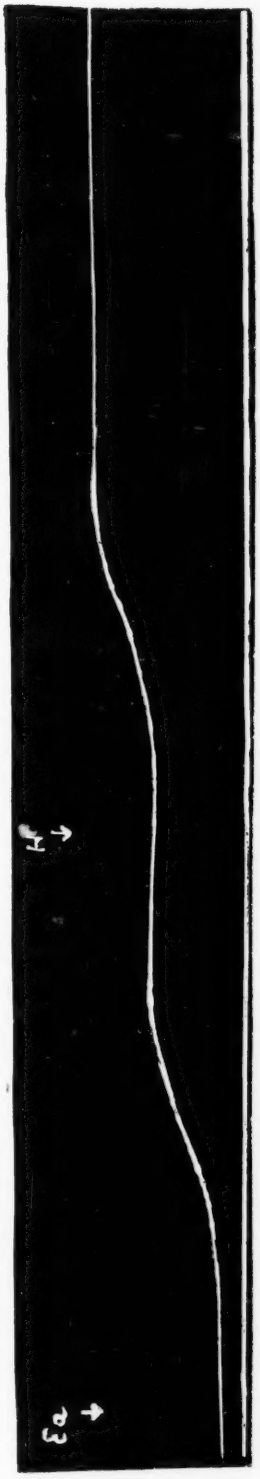

Fig. 2.—A tracheal strip from a guinea pig sensitized to egg albumin was suspended in the bath and 4 mg. of egg albumin was added. After maximal contraction, the addition of histamine, 0.1 mg. per 100 c.c., resulted in further contraction.

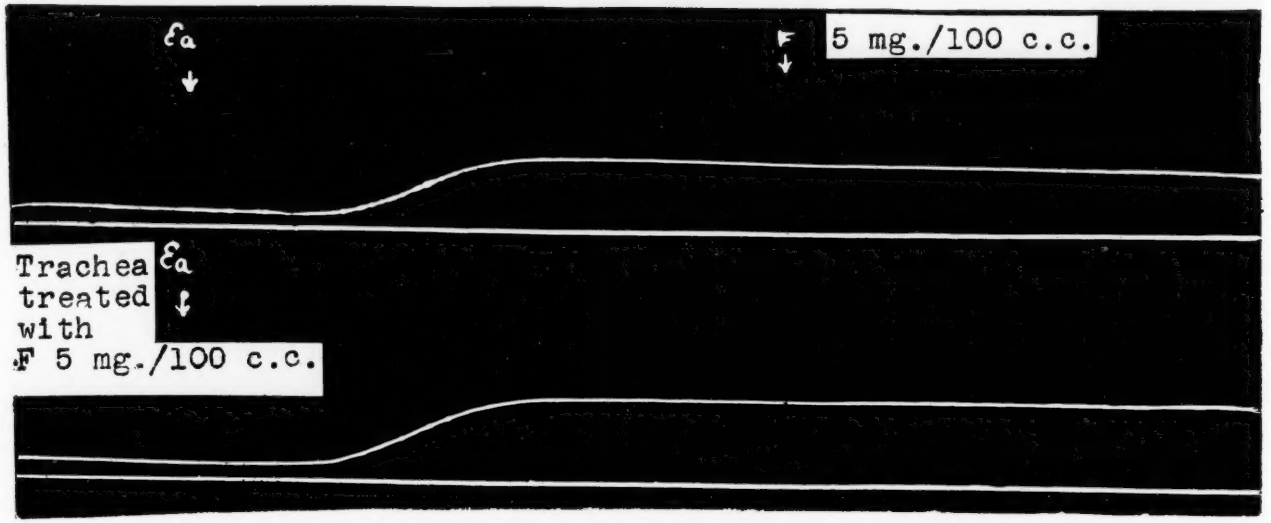

Fig. 3.—Contraction of tracheal strips from a guinea pig sensitized to egg albumin.
Above: The addition of hydrocortisone, 5 mg. per 100 c.c., did not alter the contraction.
Below: Pretreatment with hydrocortisone, 5 mg. per 100 c.c., did not prevent the contraction.

Fig. 4.—Contraction of a tracheal strip with 4 mg. of egg albumin. The trachea was from a guinea pig passively sensitized with rabbit antiegg-albumin serum.

In Vivo Passive Sensitization of the Guinea Pig Trachea With Rabbit Antiegg-Albumin Serum.—Serum from a rabbit sensitized with egg albumin was injected in the ventricle of a guinea pig. Approximately 300 meg. of antibody nitrogen were used. Fourteen hours later egg albumin was added to the guinea pig trachea suspended in the bath. No contraction occurred. The same procedure was carried out forty-eight hours after injection of the rabbit antiegg-albumin serum, and contraction resulted following the addition of 4 mg. of egg albumin to the bath (Fig. 4).

Failure to Produce Reverse Passive Anaphylaxis With the Guinea Pig Trachea.—Egg albumin, 7.5 mg., was injected intraperitoneally into a normal guinea pig. Forty-eight hours later the guinea pig trachea was suspended in the bath and rabbit antiegg-albumin serum, containing approximately 300 meg. f antibody nitrogen, was added. No contraction occurred.

Failure of In Vivo Sensitization With Guinea Pig Antiegg-Albumin Serum.—The experiment described in the discussion of in vivo passive sensitization of the guinea pig trachea with rabbit antiegg-albumin serum was repeated, using guinea pig antiegg-albumin serum instead of rabbit serum. No contraction resulted.

Failure of In Vitro Passive Sensitization of Guinea Pig Trachea.—A normal guinea pig trachea was incubated at 37° C. for three hours in serum from a guinea pig sensitized to egg albumin. Following this incubation period, the trachea was suspended in Ringer-Locke solution and egg albumin was added to the bath. No contraction of the trachea occurred. This experiment was repeated with rabbit antiegg-albumin serum and with human serum from a patient sensitive to ragweed pollen. The addition of egg albumin to the former and ragweed pollen extract to the latter did not result in a contraction.

Failure of In Vivo Passive Sensitization of Guinea Pig Trachea With Human Serum.—In this experiment 3 c.c. of human serum from a patient allergic to ragweed pollen was injected into the ventricle of a guinea pig. Forty-eight hours later the trachea was suspended in the bath and ragweed pollen extract, 1 c.c. of a 1:50 solution, was added without producing a contraction of the trachea.

DISCUSSION

The "tracheal strip" modification of the tracheal chain presents certain advantages in its preparation and use. This modification was used by Rosa and McDowell[5] in 1951 on human bronchi. The advantages in ease of preparation were recognized by these authors, but they stated that the small human bronchi used in their work were too short to provide an adequate strip preparation and the chain type of preparation seemed better suited for their study of human bronchi. Preparation of the tracheal chain requires the suturing together of ten to twelve small tracheal segments. Particularly with tracheal segments from the guinea pig, this arrangement is time consuming and difficult. It has proved to be far easier and less tedious to cut the trachea into the thin strips described above. In addition, the use of the tracheal strip results in a

greater degree of sensitivity in each individual preparation. The tracheal chain measures the change in diameter of the individual tracheal segment with each contraction. The tracheal strip measures the change in circumference of the same segment. Thus, the measurable change with each contraction is increased by π, or approximately three times, when the tracheal strip is used. In reality, however, this degree of increased sensitivity is not completely obtainable, for the tracheal strip is not stretched entirely straight and stretching it straight would result in excessive weight on the opposing side of the writing lever. Nevertheless, a definite increase in sensitivity is obtainable and this makes possible a third advantage of this modification.

The tracheal strip may be sectioned into two or three equal lengths so that additional preparations may be made from the same animal for controls or additional experiments. In the experiments reported here this feature was of great value in comparing the response of tracheal segments to histamine stimulation and antigen antibody reactions before and after treatment with hydrocortisone.

The tracheal strip preparation gave the expected response to histamine, Benadryl, and epinephrine with the normal trachea. The contraction of the sensitized trachea with the addition of egg albumin has also been reported. Contraction of the trachea with histamine following maximal contraction with antigen was not expected. This shows that the ability to respond to histamine has not been exhausted by the maximal antigen-antibody response. This is of interest in that it opposes one theory offered to explain the failure of antihistamines to antagonize some antigen-antibody reactions in human beings and in some experimental animals by the histamine theory. The explanation proposed[2] is that the concentration of histamine at the reactor site is extremely high—so high in fact, that the antihistamine levels used experimentally are not sufficient to inhibit this amount of histamine at the reactor sites. Since, however, further contraction occurs with histamine after maximal contraction with antigen, this suggests that the concentration of histamine liberated at the receptors is not as high as that administered in the experiment and failure of antihistamines to antagonize the antigen-antibody reactions must be explained on another basis.

The finding that the contraction with histamine or egg albumin of the sensitized trachea was not inhibited by hydrocortisone in the varying concentrations used is in accord with observations that hydrocortisone does not prevent anaphylactic shock or interfere with antigen-antibody reactions clinically or experimentally. Inhibition of histamine contraction of tracheal chains with hydrocotisone hemisuccinate has been described by Lefcoe,[3] and total inhibition occurred at levels of 100 mg. per 50 c.c. of solution. Even the concentrations used in the present experiments exceeded physiologic concentrations, however, and the high concentrations used by Lefcoe may have altered the response of the trachea by a "toxic" or unphysiologic mechanism.

In vivo passive sensitization of guinea pig trachea with rabbit antiegg-albumin serum is of interest. Similar responses have been noted in the guinea

pig uterus.[4] Other attempts at in vitro or in vivo passive sensitization failed under the conditions of the experiments. However, the latter failures may be due to the techniques used, and passive sensitization of the trachea may prove a valuable experimental tool.

SUMMARY

1. A modification of the tracheal chain preparation is described. This modification is easier to prepare, it is more sensitive, and it can be used in comparative studies using the trachea of one animal.

2. The response of tracheal muscle to various drugs is described. Further contraction occurs with histamine after maximal contraction with antigen. Hydrocortisone hemisuccinate did not relax or prevent tracheal muscle contraction from histamine or egg albumin in a sensitized guinea pig.

3. Guinea pig trachea may be passively sensitized in vivo with rabbit anti-egg-albumin serum.

REFERENCES

1. Castello, J. C., and de Beer, E. J.: The Tracheal Chain: A Preparation for the Study of Antispasmodics With Particular Reference to Bronchodilator Drugs, J. Pharmacol. & Exper. Therap. **90**: 104, 1947.
2. Ciba Foundation Symposium on Histamine, Boston, 1956, Little, Brown & Company.
3. Lefcoe, N. M.: The Effect of Hydrocortisone Hemisuccinate on Tracheal Smooth Muscle of the Guinea Pig and Cat, J. ALLERGY **27**: 352, 1956.
4. Kabat, E. A., and Mayer, M. M.: Experimental Immunochemistry, Springfield, Ill., 1948, Charles C Thomas, p. 149.
5. Rosa, L. M., and McDowell, R. J. S.: The Action of the Local Hormones on the Isolated Human Bronchus, Acta allergol. **4**: 293, 1951.
6. Smith, B. J.: In Vitro Action of Cortisone on Vasoconstriction due to Histamine, Proc. Soc. Exepr. Biol. & Med. **77**: 534, 1951.
7. Hawkins, D. F., Herxheimer, H., and Schild, H. O.: Responses of Isolated Human Bronchial Chains, J. Physiol. **113**: 26, 1951.
8. Hawkins, D. F., and Schild, H. O.: Action of Drugs on Isolated Human Bronchial Chains, Brit. J. Pharmacol. **6**: 682, 1951.
9. Bhattacharya, B. K.: A Pharmacological Study of the Effect of 5-hydroxytryptamine and Its Antagonists on the Bronchial Musculature, Arch. internat. de pharmacodyn. **103**: 357, 1957.
10. Bhattacharya, B. K., and Atanackovic, D.: On the Pharmacology of Procaine and Its Antagonists on the Bronchial Musculature, Arch. internat. de pharmacodyn. **104**: 3, 1956.

ALLERGENIC FOOD EXTRACTS. I. METHODS OF PREPARATION

Results of a Questionnaire

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THE Committee on Food Allergy decided to investigate the various methods by which allergenic food extracts are prepared. There were many reasons for undertaking this type of inquiry. One of the more important was the need for bringing the subject up to date by outlining the numerous methods currently in use for preparing food extracts. In addition, many of those in charge of preparing extracts have developed individual refinements which have never found their way into the literature. It was hoped that this survey might bring such refinements to light. The committee further planned to study by passive transfer techniques with reagenic sera the effect of these variables on the allergenicity of the final extract. It was hoped that the knowledge thus uncovered would enable us to prepare more uniform and active allergenic food extracts.

The present communication deals only with the information compiled from questionnaires which were sent to all members of the American Academy of Allergy residing in the United States. Without their support and splendid cooperation, this study would not have been possible, and the Committee wishes to acknowledge their aid at this time. No attempt will be made in this report to evaluate critically the many variations of procedure.

The initial postcard questionnaire sought to determine only those allergists who prepared some or all of their food extracts. A second and more extensive questionnaire (Fig. 1) was then sent to the 151 allergists who designated that they made some or all of their food extracts. Their replies to this questionnaire form the basis of our report.

All allergists who obtained extracts from a common source were treated as a unit. The twenty-four allergists in this category reduced the number preparing extracts from 151 to 127. Their answers to the fifteen questions in the circularized questionnaire are herewith summarized.

The sources used for food extracts are shown in Chart 1. Eighty-nine allergists used fresh uncooked food; sixty-six used this source exclusively, while the rest also used other sources. Only thirteen used frozen foods; two used fresh cooked food; and six used concentrated processed foods, none exclusively. Fourteen purchased defatted and dried powdered foods from allergy supply houses and, of these, six used this source exclusively.

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1. Source of raw material
   a. Fresh uncooked food in season
   b. Frozen food
   c. Fresh cooked food in season
   d. Defatted and dried powdered foods from allergy supply houses
2. Do you grind your foods? ------yes ------no
   a. Waring Blendor
   b. Juicer
   c. Scissors
   d. Meat grinder
   e. Grain grinder
3. Do you defat? ------yes ------no With EE, C, A, T, B, or S*
   a. Fruits and vegetables ----------
   b. Meats, nuts, and fish ----------
   c. Grains and spices ----------
4. Which extraction fluid do you employ?
   a. Alkaline extracting fluid (Coca's)
   b. Buffered saline extracting fluid (B.S.)
   c. Buffered sodium formaldehyde sulfoxylate (S. F. S. O.)
   d. Physiologic saline
   e. Tenth normal sodium hydroxide
   f. Sodium bicarbonate solution
   g. Glycerin saline solution
   h. Alcohol saline solution
   i. Dextrose solution
   j. Dextrose saline solution
5. Are extracts filtered? ------yes ------no
   a. Cotton pledget in filter
   b. Coarse filter paper
   c. Buchner suction funnel
   d. Centrifugation and decanting of supernatant
6. Which extracts are dialyzed?
   a. Fruits ------yes ------no
   b. Vegetables ------yes ------no
   c. Meats ------yes ------no
   d. Fish ------yes ------no
   e. Grains ------yes ------no
   f. Nuts ------yes ------no

   Dialysate (Check: H_2O------, B.S. ------, S.F.S.O. ------, etc.)

   How long are extracts dialyzed?
   1. 24 hours
   2. 48 hours
   3. Until dialysate has maintained its initial pH
7. Which extracts, if any, are concentrated?
   a. Fruits
   b. Vegetables
   c. Grains and spices
   d. Meats, fish, and nuts

   If yes, by:

(Continued on opposite page.)

*EE, ethyl ether; C, chloroform; A, acetone; T, toluene; B, benzene; S, Sovasol (Socony Mobil Oil Company, 352 North Henry St., Brooklyn, New York).

1. Evaporation through cellophane
2. Silica gel
3. Others—specify

8. How are extracts sterilized?
   a. Seitz filtration
   b. Berkefeld filtration
9. What sterility tests are used? What culture media?
   a. Culture for ------days
   b. Animal (specify) ----------inoculation
10. Is the pH of the extract checked or corrected after sterilization of extract? ------yes ------no
11. What method of standardization do you employ?
    a. Weight by volume
    b. Total nitrogen
    c. Protein nitrogen
    d. Others—please specify
12. How is your testing strength determined?
13. How often do you change your food extracts? (Testing strength)
14. How long do you think the concentrate retains its activity?
15. What is the oldest concentrate you have? To which food?

Informant—PLEASE PRINT

Address

City and State

Fig. 1.—Second questionnaire, sent to 151 members of the American Academy of Allergy.

Everyone ground the food in one way or another before extraction, except those (fourteen) who purchased the defatted and dried powdered foods as the source of their raw materials.

Table I shows that seven did not defat any foods at all; this does not include those who bought defatted dried foods. The majority, or sixty-nine, did not defat fruits and vegetables. Those who did (twenty-five) used ethyl ether in fifteen instances, toluene in six, sovasol in two, and acetone in two. Only fourteen did not defat the meats, fish, and nuts. The other ninty-eight defatted with

TABLE I. NUMBER OF PHYSICIANS WHO DEFATTED OR DID NOT DEFAT FOODS

DEFATTING AGENT	FRUITS AND VEGETABLES	MEATS, FISH, AND NUTS	GRAINS AND SPICES
Ether	15	60	47
Acetone	2	16	8
Toluene	6	13	10
Sovasol	2	7	7
Petroleum ether or trichlorethylene ether	0	2	2
Total defatting	25	98	74
Not defatting	69	14	26

There were seven who defatted no foods. Those who purchased defatted and dried foods are not included in the above table.

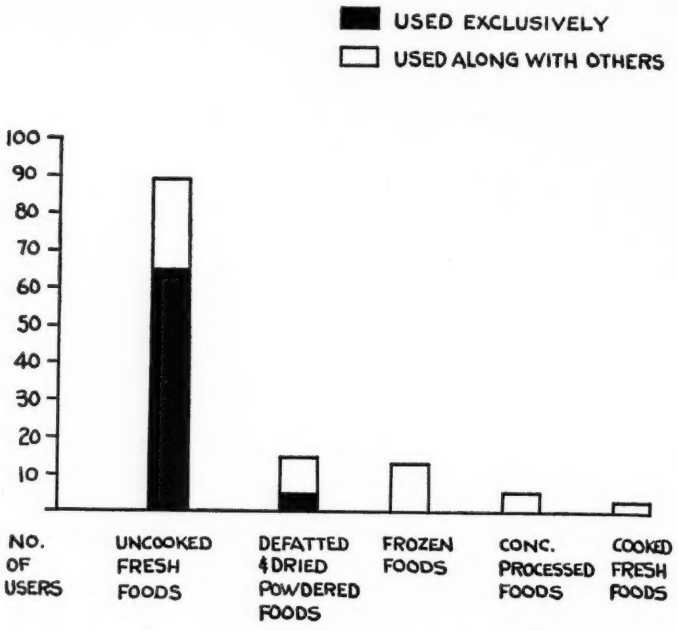

Chart 1.—Sources of foods.

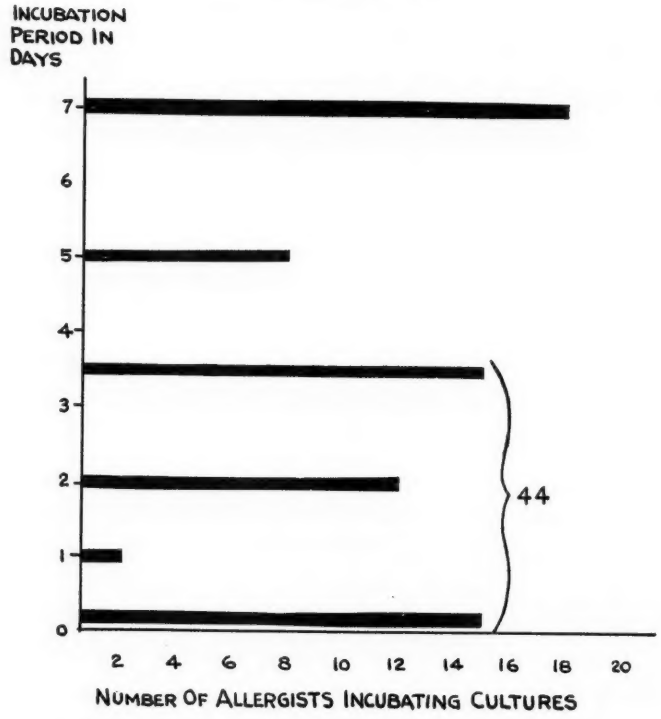

Chart 2.—Incubation of cultures for sterility tests.

ethyl ether in 60 instances, with toluene in thirteen, with acetone in sixteen, with sovasol in seven and one each with petroleum ether and trichlorethylene. Twenty-six did not defat the grains and spices. Of the seventy-four remaining, forty-seven defatted with ethyl ether, ten with toluene, seven with sovasol, eight with acetone, and one each with petroleum ether and trichlorethylene.

Table II shows that there were fifty-one allergists who used Coca's alkaline extracting fluid; of these, twenty-two used it to the exclusion of all others. Thirty-one used buffered saline (eight used it exclusively). Buffered sodium formaldehyde sulfoxylate was used nonexclusively by eleven. Physiologic saline

TABLE II. NUMBER OF PHYSICIANS USING VARIOUS EXTRACTING FLUIDS

EXTRACTING FLUIDS	USED EXCLUSIVELY	USED ALONG WITH OTHERS	TOTAL
Coca's alkaline bicarbonate	22	29	51
Buffered saline	8	23	31
Buffered S.F.S.O.*	0	11	11
Physiologic saline	1	3	4
Glycerin saline	17	23	40
Dextrose	5	3	8
Dextrose saline	2	3	5

*S.F.S.O., Sodium formaldehyde sulfoxylate.

was used by four (exclusively by one). Forty were using a glycerin saline extracting fluid (seventeen exclusively). Dextrose solution was used by eight (exclusively by five). Dextrose saline was used by five (exclusively by two).

Table III shows the number of physicians who did or did not dialyze their extracts.

The extracts were sterilized by Seitz filtration in ninety instances and by Berkefeld filtration in four. One physician stored under toluene and another added 0.5 per cent phenol to the extracting solution as the sole means of sterilizing extracts.

TABLE III. NUMBER OF PHYSICIANS WHO DID NOT DIALYZE FOOD EXTRACTS

FOOD EXTRACTS	DIALYZED	NOT DIALYZED
Fruits	34	36
Vegetables	32	36
Meats	31	36
Fish	27	39
Grains	33	35
Nuts	33	33

There were forty-six physicians who did not dialyze any food; of these, twenty-three had indicated that they used a glycerin extracting fluid so that dialysis was not practical. This group of twenty-three is not represented in the table.

There were forty-eight physicians who did not concentrate any extract. Of these, twenty-three had used glycerin as an extracting fluid and found it difficult to concentrate in this medium.

Fifteen physicians Seitz-filtered their extracts, but never checked them with sterility tests. One physician ran sterility tests only "on suspected extracts." Those remaining (seventy-one) cultured their extracts for sterility. Thioglycolate was the medium used by the majority. However, there was wide variation in the incubation period of the culture, as shown in Chart 2. While two incubated

for one day, twelve incubated for two days and fifteen incubated for three days. Eight incubated for five days and nineteen incubated for seven days or more. Only one physician used animal inoculation, and that was on a mouse.

Twenty-four physicians checked the pH of the extract before or after Seitz filtration; the majority (seventy-one) did not.

In standardizing the food extracts, sixty-nine used a weight-by-volume method of standardization, thirteen used the total nitrogen method, and twenty-three used the protein nitrogen method. Two in each group made a nitrogen determination on the fish, meats, and nut extracts, but not on the fruits or vegetables. Of the sixty-nine who used a weight-by-volume method of standardization, forty-eight did not defat the fruits and vegetables and four failed to indicate whether or not they defatted.

The variations in testing strengths on a weight-by-volume basis of standardization are shown in Table IV.

TABLE IV. TESTING STRENGTHS USED ON A WEIGHT-BY-VOLUME STANDARDIZATION

QUESTIONNAIRE NUMBER	SCRATCH TEST STRENGTH	INTRADERMAL TEST STRENGTH
61-NC*	Concentrate	Concentrate (if scratch is negative)
39-NC	Concentrate	Concentrate (if scratch is negative)
19-NC	Concentrate	1:10
106-NC	Concentrate	1:10, 1:20, 1:40, to 1:200, depending upon the food
108-C*	Concentrate	1:10
54-C	Concentrate	1:10
133-C	Concentrate	1:20
149-C	Concentrate	1:100 to 1:1,000
87-C	Concentrate	1:1,000
72-NC	1:100 (foods) 1:1,000 (nuts and fish)	1:100 (if scratch is negative) 1:1,000 (if scratch is negative)

This group represents only those who did not defat or dehydrate; each indicated a scratch and intradermal testing strength.

*NC = Not Concentrated; C = concentrated.

There was great variability in the concentrations used for skin testing. Most used a concentrated extract for scratch testing and dilutions of the concentrate for intracutaneous testing. When a concentrate was used first for scratch testing, the dilutions employed for intracutaneous testing varied from concentrate to 1:10, 1:20, 1:200, 1:1,000, and 1:5,000. This variability was further increased, when one considers that some physicians used organic solvents, such as ether or acetone, which dehydrated and defatted at the same time, thus leaving a more concentrated antigenic mass for subsequent extraction. Some extracts were concentrated further by evaporation, again increasing the variable. Those using a protein nitrogen or total nitrogen method of standardization were in closer agreement as to the testing strength for their food extracts. In general, protein nitrogen Cooke units were used in strengths of 3,500 units for fruits and vegetables, 100 units for fish, nuts, and egg, and 50 units for mustard. Total nitrogen of 0.01 to 0.001 mg. per cubic centimeter were used for the egg, mustard, and nut group, while the fish, meat, and cereal groups were diluted to 0.1 mg. per cubic centimeter.

Table V shows the consensus of opinion as to the duration of allergenic activity of concentrated food extracts. The results have been tabulated into two groups, those using various aqueous extracting fluids and those using 50 per cent glycerin. More than one-half agreed that the activity did not exceed two years. On the other hand, two thirds of those using 50 per cent glycerin as an extracting fluid felt that their extracts remained active for many years, with estimates extending from fifteen years to an infinitude. There was no inquiry into the duration of activity of food extracts in the skin-testing strengths.

TABLE V. ESTIMATES OF DURATION OF ALLERGENIC ACTIVITY OF FOOD EXTRACTS

LENGTH OF ACTIVITY	FOOD EXTRACTS			
	AQUEOUS		50 PER CENT GLYCERIN	
1-2 months	1		0	
6 months	4		1	
1 year	11		2	
1-2 years	6		0	
2 years	6	Subtotal 28	3	Subtotal 6
3 years	2		0	
Several years	4		3	
2-5 years	1		0	
5 years	4		2	
7 years	1		0	
10 years	1		3	
15 years	0		3	
Many years	0		1	
Indefinitely	0	Total 41	3	Total 21

Only those using 50 per cent glycerin exclusively as an extracting fluid were summarized for these data.

Several respondents mentioned specific variations of the regular procedures, which they felt were refinements in technique. These deserve mention at this point. One allergist concentrated his extracts with high vacuum over low temperatures. He was also experimenting with ultrasonics as a means of sterilizing extracts. Another precipitated the crude extract with acetone at -20° C. and a pH of between 4.5 and 4.7. The resultant precipitate was dried and redissolved in glycerosaline and Seitz filtered. Extracts were then made up on the dry weight basis of the precipitated protein and diluted 1:1,000 for intracutaneous and 1:50 for scratch testing. A third concentrated his extracts with the use of carbon dioxide, acetone, and a dehydrating machine. A fourth precipitated the original extract in a deep freeze with acetone. The precipitate was then filtered, washed, and dried in vacuum. The resultant powder was then milled and prepared as a 3 per cent extract in a mixture of equal parts of buffered saline and glycerin; the pH was kept constant at 7.4 without the use of dialysis. One boiled the fresh fish in water and standardized the resulting solution in concentrations of 0.02 mg. N./c.c. for skin testing. The fish itself was discarded. Finally, one allergist tested with blood sera to detect sensitivity to meats of the corresponding species.

SUMMARY

The majority of allergists making their own food extracts used fresh uncooked food as the source of their raw material. The food was then ground

or cut into smaller particles. The majority did not defat fruits and vegetables, but did defat meats, fish, nuts, grains, and spices. The majority used a weight-by-volume method of preparing their extracts and made dilutions of same for intracutaneous testing.

Either alkaline extracting fluid (Coca's solution), glycerin saline, or buffered saline was used by the allergists in general for extracting purposes.

Almost one third (forty-eight) of the allergists did not concentrate food extracts. Among these were twenty-three who used 50 per cent glycerin as an extracting fluid, which rendered it difficult to concentrate.

Nearly all physicians sterilized their extracts by filtration through a Seitz type of pad. Several allergists did no sterility tests subsequent to Seitz filtration. Twenty-nine physicians incubated for intervals up to three days.

Three-fourths (seventy-one) of the physicians reporting did not check the pH of the extract before or after Seitz infiltration. Most of these physicians did not dialyze the extract for pH adjustment.

A weight-by-volume method of standardization for food extracts was used by a majority (sixty-nine) of the allergists. There were variations in procedure in that some used a dry weight and others a wet weight as the basis for the weight-by-volume method of standardization. A few concentrated their extracts, but most did not. The strength of the testing solution showed wide variations, even among persons using the same method of extraction and standardization.

The numerous variables that have been presented are not being subjected to critical analysis. Further investigation is necessary to determine the effect of each of these variables upon the allergenicity of the final extract.

303 East 20th St. (M. B. S.)

Editorial

FOURTEENTH ANNUAL MEETING OF THE AMERICAN ACADEMY OF ALLERGY PHILADELPHIA, PA.

Feb. 3, 4, and 5, 1958

THE scientific session included, in the span of two and one-half days, four lectures by invited guests and twenty-eight papers. It was well attended by an alert and interested audience. The content of the program was too varied to permit general characterization, except the need for grateful acknowledgment of the active participation of an unusually large number of pediatricians. At least one trend was evident, namely, the increasing adoption of the "double blind" technique in the appraisal of therapeutic agents. There is little question that many a cherished "clinical impression" is doomed to wither in the chilly blast of this rigid and impersonal experimental method. Some felt that too little time was allowed for discussion of papers, a criticism to which the Program Committee might well address itself in the coming year. The standard of presentation was excellent, and the chairmen of the several sessions were surprised to find how little they had to draw on their fund of tact to meet the awkward challenge presented by the essayist who, in his worthy preoccupation, disregards the little green and red lights.

A postgraduate course, likewise very well attended, fully occupied the two days immediately preceding the scientific session. The subjects covered were allergenic extracts, steroid therapy, antibiotics in sinopulmonary disease, dermatologic and pediatric allergy, cardiac asthma, and collagen disease.

No report of a medical meeting can convey to those who were absent the rewards which accrued from the re-establishment of old friendships and from the lively discussions, to be found nowhere in the program, by members and guests collected in twos and threes in the hallways, the corridors, the elevators, and perhaps even the bars. The entire meeting was colored by the warmth and outgoing hospitality accorded the Academy by the Philadelphia Allergy Society, which deserves the Academy's heartfelt thanks and appreciation.

Announcements

Third International Congress of Allergy

The Third International Congress of Allergy, sponsored by the International Association of Allergology and French Allergy Association, will be held in Paris, France, Oct. 19 to 26, 1958.

The program will include the following:

(1) Symposia on asthma and emphysema, immunology, recent clinical advances, biochemical aspects, autoimmune reactions, dermatology, and socioeconomic aspects. Participants are world authorities in special fields—Pasteur Vallery-Radot, Löffler, Grabar, Dixon, Chase, Coombs, Forsham, Sir Henry Dale, Halpern, Schild, Harrington, Ackroyd, Dausset, Sulzberger, Jadassohn, and many others.

(2) Sectional papers, limited to ten minutes, on any phase of allergy.

(3) Round-table small-group luncheon conferences on selected subjects led by international authorities.

(4) Pre- or postconvention tours.

(5) A very enjoyable social program has been arranged.

For registration and Congress information, write to Dr. B. N. Halpern, 197 Boulevard St. Germain, Paris VII, France.

For participation on program, write to Dr. S. M. Feinberg, 303 E. Chicago Ave., Chicago 11, Illinois.

For travel and pre- or postconvention tours, write to Thomas Cook and Son, 2 Place de la Madeleine, Paris, France.

National Jewish Hospital at Denver to Choose Chief of Asthma Service

National Jewish Hospital at Denver invites applications for the new position of Chief of Asthma Service.

This institution is a 325-bed, free-care medical center for treatment, research, education, and rehabilitation in diseases of the chest. It is nonsectarian with respect to admissions and employment policies.

The asthma program calls for long-term hospitalization. It embraces the physiologic, allergy, and psychiatric aspects of both pediatric and adult intractable asthma. It aims to return the patient to his home and community able to function effectively.

The Chief of Asthma Service will have major responsibility for the clinical and research phases of this program. Therefore, he must have had broad experience in the treatment of asthma and he must have demonstrated an interest in investigation. All replies will be considered confidential. Send curriculum vitae to and list of publications to:

National Jewish Hospital at Denver
3800 East Colfax Ave., Denver 6, Colorado
Attention: Secretary.

Connecticut State Allergy Society

The Connecticut State Allergy Society met on Nov. 14, 1957, in Hartford, Connecticut. New officers for 1958 are as follows:

President: Joseph Weiner, New Haven
Vice-President: Maurice Hillman, New Haven
Secretary-Treasurer: Marvin Mogil, New Haven

International Symposium

The Mechanisms of Hypersensitivity

March 27, 28, 29, 1958

(Auspices of Henry Ford Hospital, Detroit, Michigan)

Meetings to Be Held in Auditorium
Henry Ford Hospital, 2799 W. Grand Blvd., Detroit, Mich.

PROGRAM COMMITTEE

Chairman:

Merrill W. Chase
The Rockefeller Institute of Medical Research
New York City, New York

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Walter J. Nungester
University of Michigan
Professor and Chairman
Department of Bacteriology
Ann Arbor, Michigan

John W. Rebuck
Division of Hematology
Henry Ford Hospital

GENERAL DISCUSSION

Material presented in general discussions will be without lantern slides unless cleared one day in advance of the meeting with the Symposium Committee.

PROGRAM

March 27, 1958—Thursday, A.M.

First Morning Session

8:50 A.M. Remarks of Welcome — Robin C. Buerki, Executive Director, Henry Ford Hospital

8:55	Announcements	Joseph H. Shaffer

Chairman: Merrill W. Chase, New York City

Heterogencity of Antibodies

9:00	Chairman's remarks	Merrill W. Chase
9:05	Evidence for Qualitative Differences in Antibodies	David W. Talmage Chicago
9:30	Quantitative Measurement of Heterogeneity Among Antibodies	Fred Karush Philadelphia
9:50	Heterogeneity of Diphtheria Antitoxins	Marcel Raynaud Garches, France
10:10	Discussion designated	William C. Boyd Boston William Joseph Kuhns Pittsburgh William H. Taliaferro Chicago
10:30	General discussion	
10:45	Intermission	

Chairman: Bram Rose, Montreal

Detection of Antibodies in Human Sera

11:00	The Detection of Non-Precipitating Antibodies in Allergic Sera	Alec H. Sehon Montreal, P.Q.
11:15	Detection of Antibody Binding a Component in Ragweed Pollen Extract	Francis C. Lowell Boston
11:30	Detection of Human Blocking Antibody to Ragweed by Inhibition of a Complement Fixation Test	William B. Sherman and Joseph Portnoy New York City
11:40	Approaches to the Problem of Detecting Antibodies	Stephen V. Boyden Copenhagen, Denmark
12:05	Discussion designated	Dan Hampton Campbell Pasadena
12:10	General discussion	
12:30	Luncheon	

March 27, 1958—Thursday, P.M.

First Afternoon Session

Chairman: Walter J. Nungester, Ann Arbor

Effects of Antibody and of Antigen–Antibody Complexes on Intact Cells and Whole Organisms

2:00 P.M.	Some Cytologic Effects of Antibodies	Harrison Latta Los Angeles
2:15	Discussion designated	John W. Rebuck Detroit
2:25	In Vivo Localization of Specific Anti-Organ Sera: Relation to Occurrence of Renal Lesions	Beatrice Carrier Seegal New York City
2:35	Role of Antibody and of Antigen–Antibody Complexes in Experimental Hypersensitivity	Frederick G. Germuth, Jr. Baltimore
3:00	Role of In Vivo Antigen–Antibody Precipitation in Hypersensitivity Reactions	George Edward McKinnon Salt Lake City
3:10	Intermission	
3:30	An Antigen-like Substance Obtained From Serum After Disappearance of the Original Antigen	Ludwig A. Sternberger Army Chemical Center, Maryland

3:45	Immunohistochemical Analysis of Hypersensitivity and Related Lesions	Frank James Dixon, Jr. Pittsburgh
4:05	Discussion designated	Charles A. Janeway Boston Victor A. Najjar Nashville, Tenn.
4:25	General discussion	

First Evening Session

Chairman: Frank C. MacIntosh, Montreal

Permeability Factors

8:00 P.M.	Chairman's remarks	Frank C. MacIntosh
8:10	Serotonin	Sidney Udenfriend Bethesda, Maryland
8:35	Histamine Metabolism	Richard W. Schayer Rahway, N. J.
9:00	The Effect of Corticosteroids Upon In Vitro Blood Histamine Release	Joseph W. Noah St. Louis
9:10	Mechanisms of Hypersensitivity as Revealed by the Actions of Inhibitors	H. O. Schild London, England
9:40	General discussion	

March 28, 1958—Friday, A.M.

Second Morning Session

Chairman: Irwin H. Lepow, Cleveland

Participation of Complement in Allergic Responses

9:00 A.M.	Complement: a Review (Including Esterase Activity)	Irwin H. Lepow
9:20	Dependency of Passive Cutaneous Anaphylaxis on Complement	Abraham G. Osler Baltimore
9:35	In Vitro Models for the Allergic Reaction	Elmer Lewis Becker Washington, D. C.
9:50	Discussion designated	Herbert Rapp Baltimore Otto J. Plescia New Brunswick, N. J.
10:05	General discussion	
10:20	Intermission	

Chairman: Frank L. Adler, New York

Auto-antibodies

10:40	Chairman's remarks	Frank L. Adler
10:45	Autoimmunity in Thyroid Diseases	I. M. Roitt and D. Doniach London, England
11:05	Autoimmune Hemolytic Anemias	Scott Swisher Rochester, N. Y.
11:25	Anti-Nuclear "Antibodies" in Lupus Erythematosus	Halsted Ried Holman New York City
11:40	Research on L.E. Cell Formation (Motion Picture)	Roger Robineaux Paris, France
12:05	Discussion designated	Stuart C. Finch New Haven
12:20	General discussion	
12:30	Luncheon	

March 28, 1958—Friday, P.M.

Second Afternoon Session

Chairman: Herman N. Eisen, St. Louis

Delayed Type Hypersensitivity Reactions

2:00 P.M.	Chairman's remarks	Herman N. Eisen
2:05	Relationship of the Delayed Hypersensitive State to Antibody Formation	A. M. Pappenheimer, Jr. New York City
2:25	The Cellular Response in Forms of Delayed and Immediate-Type Skin Reactions in the Guinea Pig	Melvin H. Kaplan, Cleveland and Louis Dienes, Boston
2:40	Discussion designated	Samuel B. Salvin Hamilton, Montana
2:50	Transfer of Bacterial Hypersensitivity in Man	H. Sherwood Lawrence New York City
3:05	General discussion	
3:15	Intermission	
3:30	Agammaglobulinemic Humans and Delayed-Type Skin Reactions	Robert A. Good Minneapolis
3:50	Metabolic Activities of Isolated Intact Lymph Node Cells	Herman N. Eisen St. Louis
4:05	Histamine in Allergic Responses of the Skin	Theodor Inderbitzin Zurich, Switzerland
4:25	General discussion	

Second Evening Session

Hotel Statler

6:30 Reception

7:30 Banquet

Chairman: Geoffrey Edsall, Washington, D. C.

Speaker: Are We Too Trigger Happy?? Arnold Ashley Miles, London, England

March 29, 1958—Saturday, A.M.

Third Morning Session

Chairman: Frederick G. Germuth, Jr., Baltimore

Immunologic Unresponsiveness

9:00 A.M.	Immunologic Unresponsiveness to Allergenic Chemicals	Merrill W. Chase
9:15	Further Observations on Immunologic Unresponsiveness Induced by Type I Pneumococcal Polysaccharides	Orton K. Stark Oxford, Ohio
9:25	Immunologic Unresponsiveness to Protein Antigens	William Oliver Weigle Pittsburgh
9:35	Competition of Antigens	Frank L. Adler New York City
9:45	Discussion designated	Felix Haurowitz Bloomington, Indiana Philip Y. Paterson Bethesda, Md., and New York City
10:00	General discussion	
10:10	Intermission	

Chairman: Chandler A. Stetson, New York City

Tolerance and Rejection of Tissue

10:25	Transplantation Immunity and Hypersensitivity	Leslie Brent London, England

10:50	The Role of Antibody in the Rejection of Homografts	Chandler A. Stetson
11:05	Acquired Resistance in Transplantation of Cells and Tissue	T. N. Harris and Susanna Harris Philadelphia
11:20	General discussion	

Chairman: Bram Rose, Montreal

Hormones and Allergic Responses

11:30	Summary of Present Status of Subject	Bram Rose
11:40	Effect of Steroids on Antibody Production In Vivo	Marvin Darrach Vancouver
12:00	Cortisone and Lymphoid Tissue in Relation to Hypersensitivity	Herbert C. Stoerk Rahway, New Jersey
12:20	General discussion	
12:30	Luncheon	

March 29, 1958—Saturday, P.M.

Third Afternoon Session

Chairman: Herbert C. Stoerk, Rahway, New Jersey

The Role of Mycobacteria in Allergic Manifestations

2:00 P.M.	Chairman's remarks	Herbert C. Stoerk
2:05	The Sensitizing and Adjuvant Effects of Mycobacterial Cells and Fractions	Robert George White London, England
2:20	The Development of Arthritis in the Rat Following Adjuvant Injection	Carl M. Pearson Los Angeles
2:45	Disseminated Granulomata in the Guinea Pig	Merrill W. Chase
2:55	The Mechanism of Experimental Encephalomyelitis as an Allergic Disease	Byron H. Waksman Boston
3:10	Discussion designated	Leon Sokoloff Bethesda, Md. Charles Ragan New York City
3:30	Intermission	

Chairman: Merrill W. Chase, New York City

Some Factors Modifying the Response to Allergens

3:45	The Hereditary Predisposition in Man to Develop Hypersensitivity—A Critical Review	Paul F. de Gara New York City
4:00	Non-hereditary Factors Affecting Allergic Contact Dermatitis in Man	Albert Montgomery Kligman Philadelphia
4:20	Discussion designated	Merrill W. Chase Davis Antony Long Pittsburgh
4:25	General discussion	

Obituaries

TOWNSEND B. FRIEDMAN
1905-1957

TOWNSEND B. FRIEDMAN died on March 6, 1957, of a coronary occlusion. He was born on Dec. 6, 1905; he received his B.S. degree from the University of Pennsylvania in 1927 and his M.D. degree from the University of Chicago in 1933. He interned at Michael Reese Hospital and served with the George Piness Group in 1935. Dr. Friedman was certified by the Board of Internal Medicine in 1940 and in the subspecialty of allergy in 1942. He was an instructor at Northwestern University Medical School, head of the Department of Allergy at Children's Memorial Hospital, and an attending physician and chairman of the Department of Allergy at Michael Reese Hospital.

He made many contributions to study, practice, and research in allergy and immunology, and his publications in these fields are of recognized merit.

He served in the United States Navy from 1941 to 1946, and at the time of his death was a captain in the Naval Reserve Medical Corps.

He is survived by his wife; a daughter, Mrs. Joseph Reich; and a son, Townsend, Jr.

Townsend B. Friedman was held in high esteem and respect by his friends and his associates. He was beloved by his patients and will be sorely missed by all who knew him.

M. M. M.

SIMON STEIN LEOPOLD
1892-1957

SIMON STEIN LEOPOLD was born on March 21, 1892, the son of a physician, Isaac Leopold, and died on July 13, 1957, of pulmonary thrombosis. He was educated at Penn Charter School and the University of Pennsylvania, from which he received his M.D. degree in 1914. After an internship at the hospital of the University of Pennsylvania, he entered the Army Medical Corps in 1917. He was stationed at Camp Dix, where he was in charge of the Pneumonia Service throughout the first World War.

His professional career was spent in Philadephia in the practice of internal medicine. He was attached to the School of Medicine and also to the Graduate School of Medicine of the University of Pennsylvania, where he rose to the position of Professor of Clinical Medicine and became Emeritus Professor

shortly before his death. He headed the Department of Physical Diagnosis in the School of Medicine. He was physician to the hospital of the University of Pennsylvania and chief of its Thoracic Clinic.

Dr. Leopold had a deep and sustained interest in allergic disorders (notably asthma), in bronchopulmonary conditions generally, and in infectious diseases. His great strength in these fields lay in his broad interest in all phases of internal medicine. Thus, about half of his numerous publications were in those fields, while the rest covered a wide clinical range. He was a careful worker in the research laboratory as well as in the clinic. As a teacher of physical diagnosis he was outstanding. It follows that his major literary achievements include his revision of Norris and Landis' *Diseases of the Chest and the Principles of Physical Diagnosis* and finally his own textbook, *Principles and Methods of Physical Diagnosis* (Philadelphia, 1952, W. B. Saunders Company).

He was a Fellow of the American Academy of Allergy and a member of both its parent societies; he also was a Fellow of the American College of Physicians and of the College of Physicians of Philadelphia. He served for a number of years as a member of the Board of Directors of the Philadelphia County Medical Society and for one term as its vice-president.

In 1920 he married Lorraine Livingston, who survives him. He is also survived by three sons, Robert (a physician), Theodore, and George, and a brother, Charles, an engineer with whom he conducted some of his experimental work.

R. A. K.

Correspondence

To the Editor:

It is generally recognized that acute infection of the respiratory tract in asthmatic persons is often followed by a precipitation of an asthmatic attack or by a sharp exacerbation of symptoms in the chronic asthmatic, regardless of the patient's age and the duration of the allergy and also regardless of whether the allergy is periodic and paroxysmal or is persistent.

We use the term *asthmatic bronchitis* for the clinical state in which infection of the respiratory tract occurs as a primary and single etiological agent or is a complication of rhinitis or asthma due to inhaled or ingested allergens. We are not concerned here with a consideration of the nature of the reaction as based solely on an infection or as a sensitization phenomenon to bacterial or viral agents involved in the case.

For many years it has been our policy to attempt to protect and immunize patients with a history of recurring infections, usually in the late fall and winter months, by means of vaccines made from autogenous cultures as well as stock vaccines from flora of the respiratory tract. We have used separately or in conjunction with these the polyvalent types A and B influenza virus vaccine that has been available commercially.

With the report by J. A. Bell and associates[1] of the benefits obtained with use of APC virus vaccine 4-5-7, it was decided in the fall of 1955 to add this new vaccine* to our prophylactic therapy from November, 1955, to February, 1956.

This is a clinical report on the apparent value of adenovirus vaccine in a group of forty-three patients, ranging in age from 6 to 58 years, whose recurrent infections had not been adequately controlled by the above-mentioned measures over the previous three or four winters during which they had been studied. One milliliter of the vaccine was given subcutaneously without any ensuing local or general reaction in any case.

A review of the case histories in September, 1957, may be summarized as follows:

Better	25 cases
Unchanged	10 cases
Worse	2 cases
Not reported	6 cases
Total	43 cases

In view of the favorable indications, from a purely clinical point of view, a more extended and intensive clinical and immunologic study is now under way with an improved vaccine* containing types 1, 2, 3, 4, 5, and 7.

ROBERT A. COOKE AND EDWARD J. CONWAY
Institute of Allergy
Roosevelt Hospital
60 East 58th St.
New York 22, N. Y.

REFERENCES

1. Bell, J. A., Hantover, M. J., Huebner, R. J., and Loosli, C. G.: Efficacy of Trivalent Adenovirus (APC) Vaccine in Naval Recruits, J. A. M. A. **161:** 1521, 1956.
2. Evans, Alfred S.: Correspondence, J. A. M. A. **162:** 1178, 1956.

*These vaccines were supplied through the courtesy of Parke, Davis & Company.